

The inhibitory activity of *Cassia alata* leaves extract on dengue-2 replication infected mice

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Received 20 May 2022 ♦ Accepted 21 July 2022 ♦ Published 13 September 2022

Citation: Angelina M, Hanafi M, Yuliani T, Suyatna F, Soediro TM, Dewi BE (2022) The inhibitory activity of *Cassia alata* leaves extract on dengue-2 replication infected mice. *Pharmacia* 69(3): 821–826. <https://doi.org/10.3897/pharmacia.69.e86777>

Abstract

Dengue fever is widely prevalent in Indonesia and many other tropical countries. There has been no antiviral protection against DENV (dengue virus) due to the scarcity of animal models that replicate the symptoms of its infection in humans. Subsequently, Balb/c mice were used to optimize the animal models that were infected with DENV, and measurements of DENV titer, platelet count and leukocytes counts were taken after six days of CA (*Cassia alata* leaves extract) treatment to examine the inhibitory activity of viral replication on dengue-infected mice. The results showed that CA significantly reduced DENV titer and increased platelet count compared to the control group ($P < 0.05$) but had no significant effect on leukocyte counts ($P > 0.05$). Therefore, it could be implied that *Cassia alata* could be further investigated for medication against DENV, prospectively.

Keywords

DENV, Antiviral activity, *Cassia alata* extract, 2-day-old balb/c mice

Introduction

DENV (Dengue virus) infections affect over 390 million people worldwide every year, with Dengue fever increasing by 30 times in the last five years. It is estimated that 50 to 100 million new infections occur each year in more than 100 endemic countries. According to WHO, DENV infection is one of the world's top health threats. Consequently, an antiviral was designed to reduce viral titers in the early stages of the infection cycle and prevent DHF/DSS in the process (Canard 2011). It is expected to reduce viral transmission, which leads to a reduction in the number of infected vectors and the prevention of the spread of endemic DENV infection (Sampath and Padmanabhan 2009). Therefore, the

DENV antiviral is important in treating DENV infections. Numerous studies have been conducted on the development of synthetic and natural dengue antivirals, as well as isolates from natural items. The results showed that some herbal products had DENV antiviral activity by in vitro studies (El-adawi et al. 2011). Rosmalena et al. have reported an extract of *Myristica fatua*, *Cymbopogon citratus* and *Acorus calamus* to have antiviral effects to DENV with $EC_{50} < 30 \mu\text{g}/\text{mL}$ (Rosmalena et al. 2019) However, there is currently no approved treatment for DENV, primarily because of the scarcity of animal models for preclinical testing. According to journals, DENV-2 (non- neuro adapted) infected with Balb/c mice that have been administered IP and IV can provide enough information about the morphological

characteristics of DENV infection (Cruz-Oliveira et al. 2015; Salomão et al. 2018).

Cassia alata Linn is an Indonesian native plant, but it is also found in the United States, India, Malaysia, Brazil, and Africa. This plant contains biomolecules with a wide range of bioactivity, making it a potential source of medicine for many diseases. Additionally, the plant contains secondary metabolites, such as anthraquinone glycoside chemicals, polyphenols, flavonoids, and polysaccharides. Some compound that isolated from leaf *C. alata* ethanol extract were 3,5,7,4'-tetrahydroxy flavone, 2,5,7,4'-tetrahydroxy isoflavone anthraquinone and kaempferol 3-O-gentiobioside (Fatmawati et al. 2020). Several compounds identified from the leaves of *Cassia alata*, such as aloe-emodin, emodin, rein, and chrysopanol have been shown to stop the flavivirus genus, specifically the Japanese Encephalitis Virus, from spreading (Meenupriya et al. 2014).

According to previous studies on the "in vitro inhibition of DENV", the ethanol extract from *Cassia alata* can reduce DENV replication (Angelina et al. 2017, 2020). This study aimed to determine the inhibitory activity and acute toxicity of *Cassia alata*, hence, an "in vivo" test was performed on suckling mice balb/c infected with DENV.

Materials and methods

Study design

This was an experimental study that used DENV NGC serotype 2 and 2-day-old balb/c mice to investigate the role of *Cassia alata* (CA) as a DENV replication inhibitor. The procedure was reviewed and approved by the Ethical Committee No. 17-03-0303 in the Medical Faculty of University of Indonesia.

Preparation of extract

Cassia alata leaves were collected in the Puspipetek area and identified at the Botany Herbarium Research Institute in Cibinong, Indonesia. Pulverized *C. alata* leaves of 1 kg were extracted in 70% ethanol at room temperature. Subsequently, the filtrate was filtered and dried into a thick extract using a reduced pressure rotary evaporator at 37 °C. The CA thick extract was then dissolved in distilled water containing 0.5% CMC for homogeneous concentrations of 0.2, 0.4, and 1 g/kg BW.

Preparation of DENV-2 and vero cell line

The Vero cell line was cultured in DMEM (Gibco) and supplemented with 10% FBS (Gibco) and 2% Penstrep (Gibco) in minimum essential media (Gibco). Subsequently, cryo isolate DENV-2 NGC was thawed and propagated on a monolayer Vero cell line in 2% FBS (Gibco), 2% Penstrep (Gibco) in minimum essential media (Gibco) and tested for DENV titer using the Focus assay technique.

Optimization of DENV-2 infected into valb/c mice

The DENV-2 infection was conducted in twenty-four 2-day-old Balb/C mice, and these mice were infected intracerebrally with 50 µl of DENV-2 (1.8×10^5 ffu/ml). Subsequently, the serum and mice brains were collected a day after infection for eight consecutive days. The brains were washed with 1x PBS before being placed in 1 ml of 2% FBS MEM as a medium for brain preservation, and the brain of each mouse was mechanically homogenized using a dounce homogenizer on ice. The homogenate was centrifuged for 5 minutes at 12000 rpm and 4 °C. The supernatant was also collected and stored at -80 °C. Lastly, the focus assay method was used to determine the supernatant titer.

Inhibitory activity of *C. alata* extract DENV-2 infection

Twenty four of 2-day old mice were infected with 50 µl of 1.8×10^5 ffu/ml DENV-2 and divided into four groups. The first contained untreated infected mice, while the second contained infected mice treated with CA 0.2 g/kg BW. The infected mice in the third group were treated with CA 0.4 g/kg BW, while those in the fourth were treated with CA 1 g/kg BW a day after infection. Lastly, CA was administered orally for six days.

Blood and organ harvesting

The whole blood was collected on the seventh day to measure platelets and leukocytes, while the serum obtained was used to measure cytokines and determine the viral titer of the brain.

DENV-2 titration

Monolayer Vero cells were injected in 96-well plates with serially diluted brain supernatants in a culture medium. Subsequently, the cells were cultured for 48 hours in media containing 1% Methylcellulose (Sigma-Aldrich) after the virus was adsorbed for 1 hour at 37 °C. The cell was fixed with 10% formaldehyde, followed by a secondary antibody called horseradish peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich) before staining with anti-DENV human patient serum as the primary antibody. A metal Enhanced DAB Substrate kit was also used to visualize infectious foci (ThermoFisher Scientific, Rockford, IL, USA). The foci images were counted using inverted microscopes, and the titer calculation (Ffu/ml) was based on the sum of foci and the dilution factor (Angelina et al. 2017).

Platelets and leukocytes counting

The platelets and leukocytes of each animal were counted using an automatic machine hematology analyzer (Sysmex).

Results and discussion

Optimization of DENV-2 infected animals

Twenty-four infected mice were selected and divided into eight sets. Subsequently, three were killed daily, with their brains and lymph nodes harvested. The highest virus titer was found in the brain supernatant and increased daily after infection, reaching its peak on the seventh day. However, it went down on the eighth day, as shown in Fig. 1.

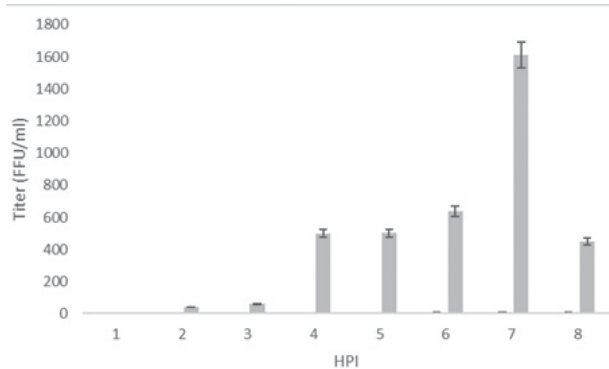


Figure 1. DENV-2 Titer in Supernatant Brain of Mice, (HPI = days post infection).

It was established that the inhibitory DENV-2 testing was conducted for seven days because the viral titer had naturally dropped by the eighth day. This result correlated with previous studies that the viral titer can be found in the supernatant of mice's brains, although the titer would naturally decrease due to the host's defense mechanisms (Oliveira et al. 2016). Even though mice were not considered an ideal host for dengue infection but the outcome showed that the brain titer in mice increased up to the seventh day.

Effect of *Cassia alata* extract on DENV-2 infected mice

Twenty-four of the 2-day old Balb/c mice were given *Cassia alata* extract for six days after infection. The brain was surgically removed on the seventh day, and blood was collected and separated into two heparinized tubes. The first tube was made for counting platelets and leukocytes, while the other was for determining cytokine levels. The results of the test, which measured virus titer in the brain supernatant of mice, showed a decrease in the concentration in the DENV-2 infected group that received CA extract compared to the DENV-2 infected group that did not receive it, as shown in Fig. 2. The DENV-2 titer was calculated by multiplying focus and the concentration by the dilution factor (Fig. 3).

Cassia alata ethanol extract inhibited the replication of DENV-2 in brain mice, which correlates with previous in vitro studies. (Angelina et al. 2017) Previous studies also show that crude polar extracts, such as ethanol, methanol,

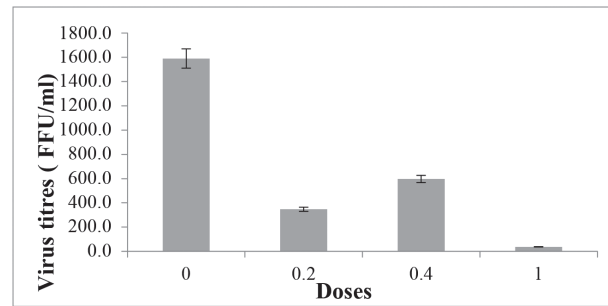


Figure 2. Titer of DENV after CA oral administration on Mice infected DENV-2 with a Variation of Doses 0, 0.2, 0.4, and 1 g/kg bw.

or water extracts, contain flavonoids and alkaloids, which inhibit a variety of flaviviruses, such as DENV, yellow fever virus, and West Nile virus (WNV) (Johari et al. 2012).

Mice (B.) Mice DENV-2 Infected + CA 0.2 g/kg bw (C.) Mice DENV-2 Infected + CA 0.4 g/kg bw (D.) Mice DENV-2 Infected + CA 1 g/kg bw, (E.) Mice DENV-2 Infected without CA extract.

An active compound in CA, known as kaempferol, has been reported to have antiviral activity present in it. Consequently, several flavonoids from kaempferol derivatives were tested for antiviral activity against human cytomegalovirus (HCMV). The most active molecules were found to be flavonoids with acyl substituents. Continuously, the antiviral properties have also been reported to be present in anthraquinone derivatives, such as aloe-emodin, emodin, and chrysophanol (Zandi et al. 2011; Mohammed et al. 2013).

The samples were checked for normal distribution before the ANOVA test was conducted. However, the value of p (<0.05), which was gotten from the Shapiro Wilk equation, indicated that the data were not normally distributed. This was corrected by transforming the data into log form to ensure normal distribution. The one-way ANOVA obtained a value of $p = 0.00$ ($p < 0.05$) from the test data, which indicated that there was a significant difference between the doses of 0.2 groups, 0.4 groups, and 1 g/kg BW with the DENV-2 infected group without CA. Further test analysis was conducted to determine which groups provided significant differences, and the test results revealed that there were significant differences in all treatment groups at doses of 0.2, 0.4, and 1 g/kg BW to the DENV-2 infected group without extracts with a p -value of < 0.05 .

Thrombocytopenia refers to a reduction in platelet count, which is caused by a decrease in production or an increase in the destruction of platelets. Platelet production is reduced in DENV infection due to suppression of megakaryopoiesis through infection of hematopoietic progenitor cells or indirectly through altered cytokine levels in the bone marrow due to impaired stromal cell function. Furthermore, platelets from dengue patients show signs of activation, mitochondrial dysfunction, and enhanced apoptosis, which may contribute to the development of thrombocytopenia. Increased platelet destruction also occurs due to platelet cross-reactivity with anti-DENV

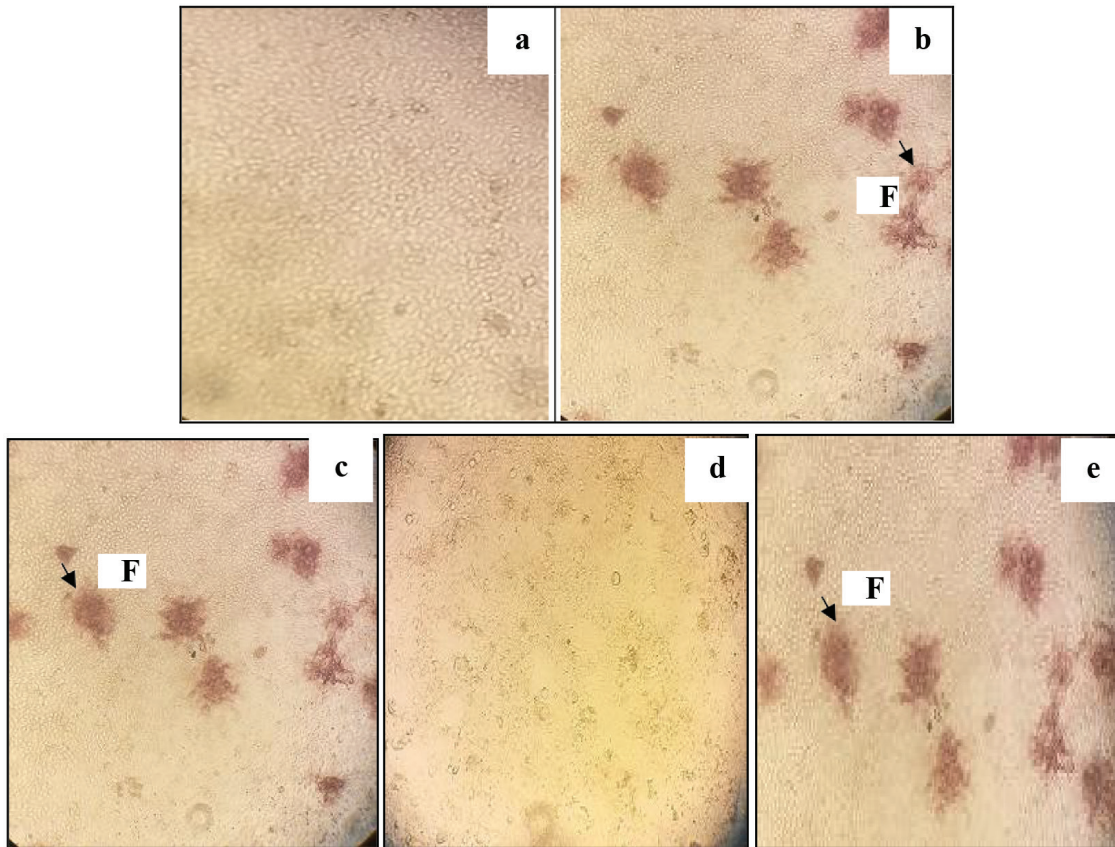


Figure 3. Immunostaining Results of the DENV-2 on focus assay. F = foccus (A) Normal.

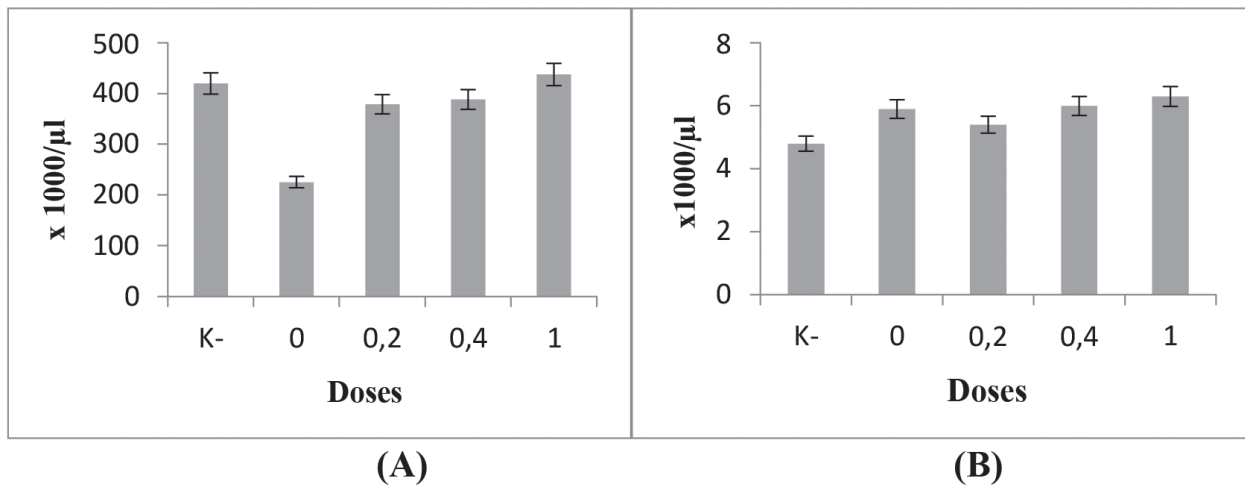


Figure 4. A. Platelet Count Between Treatment Groups Mice Infected with DENV-2 + CA Concentration of 0, 0.2, 0.4, and 1 g/kg BW
B. Number of Leukocytes Between Treatment Groups Mice Infected with DENV-2 + CA Concentration of 0, 0.2, 0.4, and 1 g/kg BW.

antibodies. Anti-non-structural protein-1 (NS-1) induced by DENV accelerates thrombocytopenia by stimulating complement-mediated lysis of platelets. NS-1 can also activate endothelial cells, increasing vascular permeability and platelet activation (Fialho et al. 2017).

The effect of CA oral administration with different doses on platelet DENV-2 infected mice showed an increase in the number of platelets, with the highest platelet count

found in mice group CA 1g/kg BW, which was significantly different from the group without administration of CA ($p < 0.05$) as shown on Fig. 4 (A). The same pattern was observed in the number of leukocytes, with the highest increase in the dose group being 0.4 g/kg BW, even though the increase showed no statistical significance when compared to the DENV-2 group without CA ($p > 0.05$) as shown on Fig. 4 (B).

Correlation analysis between viral titers and platelet counts in mice

The correlation analysis of the viral titers and platelet titer counts in mice yielded results of ($p = 0.014$; < 0.05), which indicated that there was a meaningful correlation between the number of viral titers and the number of platelets. Furthermore, a negative correlation value showed that a high viral titer was associated with a low platelet count (thrombocytopenia) as shown at Fig. 5. The correlation was classified as moderate with a value of 0.541, which indicated that the correlation between an increase in viral titer and a decrease in platelet count was moderate. Previous studies showed that DENV can cause bone marrow hypoplasia and damage to bone marrow progenitor cells during the acute phase of the disease by blocking their activity and reducing hemato-

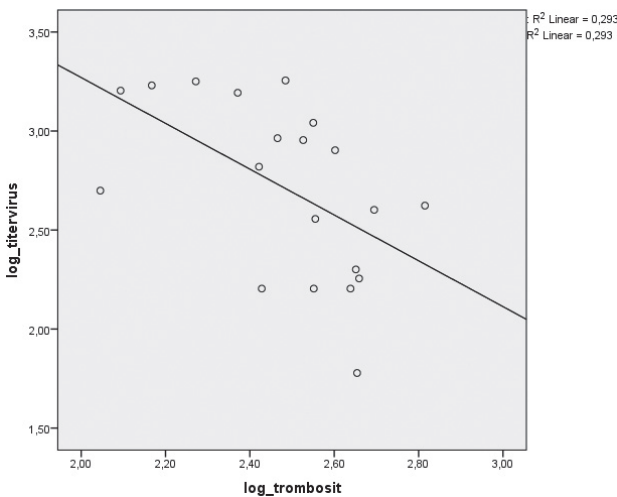


Figure 5. Correlation between the Virus Titer Log and the Platelet Log.

References

- Angelina M, Hanafi M, Suyatna FD, Dewi BE (2020) Drug of action cassia alata leaves extract as antiviral to dengue virus serotype-2 in vitro. *Pharmacognosy Journal* 12: 864–871. <https://doi.org/10.5530/pj.2020.12.124>
- Angelina M, Hanafi M, Suyatna FD, Mirawati ST, Ratnasari S, Dewi BE (2017) Antiviral Effect of Sub Fraction Cassia alata Leaves Extract to Dengue Virus Serotype-2 strain New Guinea C in Human Cell Line Huh-7 it-1. *IOP Conference Series: Earth and Environmental Science* 101. <https://doi.org/10.1088/1755-1315/101/1/012004>
- Canard B (2011) *Antiviral Research and Development Against Dengue Virus*, 101 pp.
- Cruz-Oliveira C, Freire JM, Conceição TM, Higa LM, Castanho MARB, Da Poian AT (2015) Receptors and routes of dengue virus entry into the host cells. *FEMS Microbiology Reviews* 39: 155–170. <https://doi.org/10.1093/femsre/fuu004>
- El-adawi H, El-demellawy M, El-wahab AA (2011) Some Medicinal Plant Extracts Exhibit Potency 2. *Materials and Methods. Journal of Bioscience And Technology* 2: 223–231.
- Fatmawati S, Yuliana, Purnomo AS, Abu Bakar MF (2020) Chemical constituents, usage and pharmacological activity of *Cassia alata*. *Heliyon* 6(7): e04396. <https://doi.org/10.1016/j.heliyon.2020.e04396>
- Fialho LG, Da Silva VP, Reis SRNI, Azeredo EL, Kaplan MAC, Figueiredo MR, Kubelka CF (2017) Antiviral and Immunomodulatory Effects of Norantea brasiliensis Choisy on Dengue Virus-2. *Intervirology* 59: 217–227. <https://doi.org/10.1159/000455855>
- Johari J, Kianmehr A, Mustafa MR, Abubakar S, Zandi K (2012) Antiviral activity of baicalein and quercetin against the Japanese encephalitis virus. *International Journal of Molecular Sciences* 13: 16020–16045. <https://doi.org/10.3390/ijms131216785>
- Meenupriya J, Vinisha A, Priya P (2014) Cassia alata and Cassia auriculata – Review of their bioactive potential. *World Journal of Phar-*

poietic cell proliferative ability, including platelet production (Sridharan et al. 2013).

Conclusion

In summary, *Cassia alata* leaves ethanol extract decreased the DENV-2 titer and increased platelet count significantly compared to the control group ($p < 0,05$).

Author's contributions

Author contribution statement MA: Main contributor; Performed the experiments, Data analysis: Wrote the paper. MH: Evaluation of the method and result FDS: Evaluation of the method and result TMS: Analyzed TY: Method, Editing and interpreted the data; Contributed reagents, materials BED: Analyzed and interpreted the data; Contributed reagents, materials and reviewed the paper

Funding statement

The authors would like to extend special appreciation to The Ministry of Research, Technology, and Higher Education Republic of Indonesia, specifically Sainstek Scholarship Program and DIPI LPDP for funding this study.

Declaration of interest statement

The authors declare no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

- maceutical Sciences 2: 1760–1769. <http://www.academia.edu/download/35883193/021226-PS0387.pdf>
- Mohammed MMD, El-Souda SS, El-Hallouty SM, Kobayashi N (2013) Antiviral and cytotoxic activities of anthraquinones isolated from *cassia roxburghii* linn. leaves. *Herba Polonica* 59: 33–44. <https://doi.org/10.2478/hepo-2013-0022>
- Oliveira ERA, Amorim JFS, Paes MV, Azevedo AS, Gonçalves AJS, Costa SM, Mantuano- Barradas M, Póvoa TF, de Meis J, Basilio-de-Oliveira CA, Nogueira ACMA, Alves AMB (2016) Peripheral effects induced in BALB/c mice infected with DENV by the intracerebral route. *Virology* 489: 95–107. <https://doi.org/10.1016/j.virol.2015.12.006>
- Rosmalena R, Elya B, Dewi BE, Fithriyah F, Desti H, Angelina M, Hanafi M, Lotulung PD, Prasasty VD, Seto D (2019) The Antiviral Effect of Indonesian Medicinal Plant Extracts Against Dengue Virus In Vitro and In Silico. *Pathogen* 8(2): 85. <https://doi.org/10.3390/pathogens8020085>
- Sampath A, Padmanabhan R (2009) Molecular targets for flavivirus drug discovery. *Antiviral Research* 81: 6–15. <https://doi.org/10.1016/j.antiviral.2008.08.004>
- Sridharan A, Chen Q, Tang KF, Ooi EE, Hibberd ML, Chen J (2013) Inhibition of Megakaryocyte Development in the Bone Marrow Underlies Dengue Virus-Induced Thrombocytopenia in Humanized Mice. *Journal of Virology* 87: 11648–11658. <https://doi.org/10.1128/jvi.01156-13>
- Zandi K, Teoh B-T, Sam S-S, Wong P-F, Mustafa M, AbuBakar S (2011) Antiviral activity of four types of bioflavonoid against dengue virus type-2. *Virology Journal* 8: 560. <https://doi.org/10.1186/1743-422X-8-560>