The Effectivity of Butanol Fraction of Calophyllum nodosum as Antiviral Drug to Dengue Virus Serotype 2 In Vitro

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Abstract: Dengue fever still has a high incidence rate especially in Indonesia. Until now, there is no dengue antiviral therapy found. Researches to develop dengue antiviral from herbal sources had been done. One of the potential plants as dengue antiviral is *Calophyllum nodosum* which is known to have antimicrobial activity. This study to evaluate the antiviral effects of butanol fraction of *Calophyllum nodosum* on DENV-2 activity with Huh-7-it cells as host cells and also to evaluate minimal inhibitory concentration. Antiviral capability was measured by 50% cytotoxic concentration (CC_{50}) values and 50% inhibitory concentration (IC_{50}) values. The IC_{50} value showed the effect of extract inhibition and was obtained from the focus assay of DENV after treated with serial concentrations of extract (80, 40, 20, 10, 5 and 2.5 μg/mL). The CC_{50} value showed the effect of cytotoxic extract and resulted from MTT assay using concentrations of 640, 320 , 160, 80, 40, 20, and 10 μg /mL. The selectivity index (SI) value was ratio of CC_{50} and IC_{50}. The IC_{50}, CC_{50} and SI value of butanol fraction of *Calophyllum nodosum* was 5.6 μg/mL, 1181 μg/mL and 210.9, respectively.. Statistical analysis showed significant differences between control group and treatment group on focus assay and MTT assay. It can be concluded that the butanol fraction of *Calophyllum nodosum* had strong antiviral effect with low cytotoxic effects.

1 INTRODUCTION

Dengue virus (DENV) infection is serious health problem in the world, including Indonesia. DENV is transmitted to humans by infected female *Aedes aegypti* or *Aedes albopictus* (Fatima *et al.,* 2011). There are four serotypes of dengue virus (DENV 1-4) that manifest with similar symptoms[2,3] DENV infection cause various clinical manifestation range from asymptomatic to severe cases such as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). Both DHF and DSS can cause fatal cases and can lead to death of the patients (WHO, 1997). When DENV infect to human, only few hours after infection, tens of thousands of copies of viral molecules are produced from a single viral molecule, leading to severe cases to death. Despite the availability of a dengue vaccine, improvements in case management to reduce the risk of severe dengue are still needed.

The prevention of DENV infection and better treatment have been developing. Prevention usually directed to the DENV vector control. In other hand, for DENV patient management usually given supportive care. DENV infection is self limiting disease, but there are patients with severe disease (WHO, 1997). Improvements in case management to reduce the risk of severe dengue are still needed. Current approaches are entirely supportive care in the form of judicious fluid replacement and close clinical monitoring during the critical phase of illness (WHO, 1997). Up to now, there is no specific antiviral drug to DENV even there were association between higher viremia levels and severe dengue. Development of antiviral drug to DENV may help for better treatment of DENV patients. The development of dengue antiviral drugs is still in progress. At present, the development of antiviral drug to DENV medications leads to sources of herbal medicines (Sohail *et al.,* 2011). The source of these herbal medicines is widely discovery because

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of the possibility of having low side effects and abundant in nature. Genus of Calophyllum have been widely used as traditional herbal medicines in the tropic area (Bernabé-Antonio, 2014). Those plants have phytochemicals such as flavonoids, xanthones, coumarin, chalcone, benzofuran, and triterpene. Those phytochemical have antioxidant and antimicrobial activity (Alkhamaiseh *et al*., 2012). Calophyllum plants able to inhibit the activity of bacteria and fungi (Hanafi *et al.,* 2017). Some species have also been reported to have bioactivity against various viruses such as HIV-1 virus and human leukemia HL-60 (Sánchez *et al.,* 2000). Calophyllum nodosum species also contain phytochemicals that have antioxidant and antimicrobial activity. This phytochemical content is thought to have antiviral activity against dengue virus (Hanafi *et al.,* 2017; Sánchez *et al.,* 2000). But then, the antiviral activity to DENV of *Calophyllum nodusum* has not been discovered yet. Therefore, the purpose of this study is to investigate the effectivity of leaf extract *Calophyllum nodusum* in butanol fraction as antiviral drug to DENV-2.

2 METHODS

The study was done at Department of Microbiology, Faculty of Medicine, Universitas Indonesia. We used Huh 7 it-1 cell and DENV serotype 2 strain New Guinea C. To evaluate antiviral activity of *Calophyllum nodusum*, we used previous method (Saptawati *et al.,* 2017) with slight modification. The serial dilution of extract at 320 µg/mL, 160 µg/mL, 80 µg/mL, 40 µg/mL, 20 µg/mL and 10 µg/mL were used to determine inhibition of DENV replication. To determine cytotoxic effect we used serial dilution of extract at 640, 320 µg/mL, 160 µg/mL, 80 µg/mL, 40 µg/mL, 20 µg/mL and 10 µg/mL. DMSO as a diluent of extract were used as negative control of antiviral assay. The test were made in triplicate.

2.1 Determination of half-inhibitory concentration

A total of 2×104 cells/well were seeded into 96-well plate and the plate were incubated at 37°C with 5% CO2. After 24 hours, the cells were infected with DENV-2 with MOI of 1 FFU/cell. Various concentration of extracts ranging from 320, 160, 80, 40 20 and 10, µg/mL were added shortly afterwards. After 2 hours of infection, a mixture of DMEM+2% FBS and various concentration of extracts were added with volume of 100 ul/well. The tested of each concentration were done in triplicate. Treated with 0.1% of DMSO were used as negative control of antiviral treatment. Plates were further incubated at 37°C for 3 days. Next, supernatant of viruses were harvested and determined the titter by focus assay. Briefly, 10-fold serial dilution of the supernatant was inoculated onto Huh-7 it-1 cell monolayer in triplicate wells. Absorption was carried out at 37^{o}C with 5% CO_2 for 2 hours with agitation at 30 minutes interval. Methylcellulose 1.5% overlay medium was added to the cell and incubated at 37^{o}C with 5% CO_2 for 3 days. The infected cells were stained according to previous study with slight modification (Saptawati *et al.,* 2017). First, infected cells were fixed and increased permeable for immunostaining. After cell washing, human IgG-anti dengue were added to each well 1/1000 and incubated at room temperature for 1 hour. For the secondary antibody. We used 1/1000 antihuman IgG label HRP. After washed using PBS, substrate for horseradish peroxidase were added and cells were observed for its brownish colour. Number of foci formed in each well including in negative control well was counted manually under microscope after staining. Number of foci in each treatment well was compared to that of negative control well to obtain percentage of infectivity of each well. The mean value of percentage of infectivity for each concentration triplicate was calculated and then those values were plotted against corresponding concentration to generate concentration-percentage of inhibition curve. The half-inhibitory concentration (IC_{50}) was obtained from nonlinear regression equation of concentration-effect curves.

2.2 Determination of half-cytotoxic concentration

To determine CC50, we used MTT assay as describe in our previous study (Saptawati *et al.*, 2017). MTT assay that quantified the percentage viability of Huh-7 cells after treated with a certain concentration of extract compared with DMSO 0.1%) as negative control. In 96 well flat-bottom plates (Corning, USA), cell were added as much as 2 × 10^4 cells/well and incubated at 37^{o}C with 5% CO_2 for 24 hours. Then, the cells were treated with various concentration of extract ranging from 640, 320, 160, 80, 40, 20, 10 , 5 and 2.5 µg/mL and were then incubated at 37^{o}C with 5% CO_2. After 48 hours of incubation, 20µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Promega) salt solution was added into each well and incubated for 4 hours according to the manufacturer's

instruction. Theoretical percentage toxicity of each concentration was determined by dividing the mean blanked sample optical density (ODs) by the mean blanked control ODs for each sample. The resulting percentage toxicity values of each concentration that was tested in triplicate was calculated for its mean and standard deviation and then the mean percentage was plotted to corresponding concentration to generate concentration-mean percentage of viability curve. A nonlinear regression equation was derived from the curve to calculate the half-cytotoxic concentration (CC_{50}) of each extracts.

2.3 Data Analysis

Mean difference of percentage of cytotoxicity and infectivity between treatments group and negative control was analysed using One-way ANOVA using SPSS version 23 with p value less than 0.05 ($p<0.05$) considered as statistically significant difference. The value of CC_{50} and IC_{50} were determined using simple arithmetical calculation on regression equations obtained from concentration-percentage of viability and concentration-percentage of inhibition. Then, selectivity index for each extract was derived from the ratio of CC_{50} to IC_{50}.

3 RESULTS

3.1 Percentage of DENV infectivity and IC_{50} value

After treated with extracts, the percentage of DENV infectivity in Huh 7it-1 was decrease significantly (Table 1). Addition of extract to DENV-2 at concentration of 40ug/mL and more, showed no DENV-2 in the focus assay with significantly different (Table 1), Decrease of extract concentration caused an increase of DENV infectivity. This results showed that butanol fraction of *Calophyllum nodosum* had antiviral activity to DENV-2. The infectivity value was then used to figure out an exponential regression and then to determine IC_{50}. Based on the equation, the IC_{50} value was 5.6 µg/mL (Figure 1.) with R^2 of 0.927.

Table 1 : Percentage of DENV-2 infectivity after treated with various concentration of *Calophyllum nodosum* .

Concentration (µg/ml)	Percentage of infectivity (mean% ± SD)	p Value
80	0.0 ± 0.0	0.034
40	0(0 - 2.9)	0.043
20	3.9 ± 4.5	0.046
10	41.8 ± 20.7	0.046
5	62.2 ± 17.1	0.046
2.5	52.5(37.9 - 52.5)	0.043
DMSO	99.1(99.1 - 102)	

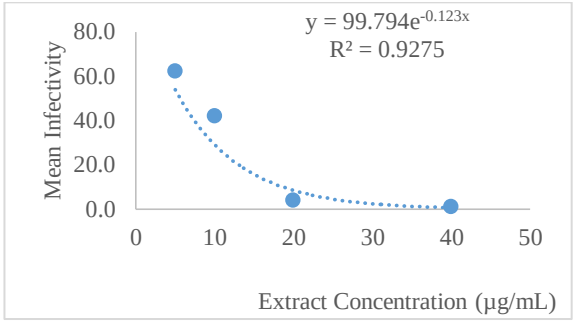

Figure 1: Exponential regression graph of DENV-2 infectivity after treated with serial concentration *Calophyllum nodosum*

3.2 Cytotoxicity and CC_{50} value

The cytotoxicity of butanol fraction of *Calophyllum nodosum* was determined by MTT assay. In MTT assay, the absorbance value of the test well divided by the absorbance value of the DMSO control, times 100% to determine the cell viability value. After treated with concentration more than 80 ug/mL, the viability of cell slightly decreased but no statistically different (Table 2). From the data, increasing of concentration of extract caused a decrease in the cell viability (Table 2). There was an abnormality data at concentration of 320 µg/mL. Treated with 320 ug/mL of butanol fraction of *Calophyllum nodosum* the cell viability decreased rapidly in excess of 640 ug/mL. It may due to a laboratory error. The mean cell viability values were then translated into a graph with a linear regression to determine CC_{50} (Figure 2). The CC_{50} value of butanol fraction of *Calophyllum nodosum* was 1,181 ug/mL with R^2 of 0.567.

Table 2 :The percentage of cell viability after treated with various concentration of extract.

Concentration (µg/ml)	Percentage of Viability (mean% ± SD)	p Value
640	82.7 ± 5.2	0.05
320	68.4 ± 5.1	0.05
160	99.7 ± 11.4	0.513
80	94.7 ± 1.1	0.05
40	102.1 ± 1.1	0.275
20	106.5 ± 0.9	0.05
10	104.9 (103.6 - 105.5)	0.05
5	101.5 ± 1.7	0.275
2.5	102.4 ± 8.6	0.827
DMSO	100.0 ± 1.7	

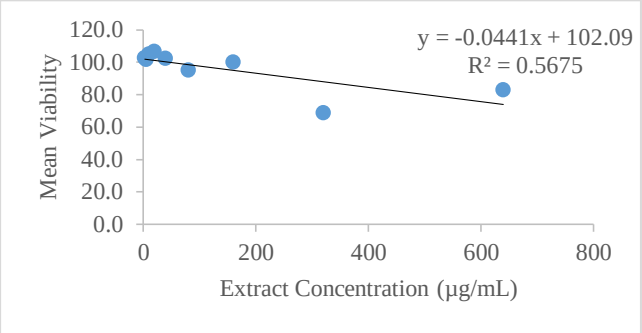

Figure 2. Linear regression graph of concentration-mean percentage of Huh7it-1 cells viability after treated with *Calophyllum nodosum*.

3.3 Selectivity Index (SI) value

The selectivity index of butanol fraction of *Calophyllum nodosum* was 210.9 based on IC_{50} and CC_{50} value.

4 DISCUSSION

Specific antiviral drug to DENV was not avalable yet. Indonesia has variety of herbal medicine that can be developed as antiviral drug to DENV. *Calophyllum* genus is known to have antimicrobial properties that can inhibit bacterial, fungal and viral activity. The phytochemical properties of the *Calophyllum* genus are flavonoid, kumarin and xanthone. Flavonoids have strong antioxidant, antimicrobial and antiviral activity. Flavonoid from other plant also contain lots of flavonoids and have the ability to inhibit viruses including DENV.[11] Several studies on the antiviral effect of Calophyllum on dengue virus have been carried out and showed that Calophyllum extract had an inhibitory effect on dengue virus activity which was significant with a relatively small cytotoxic effect.[12,13] Similar result was found in this study. Butanol fraction of *Calophyllum nodosum* showed antiviral activity with IC_{50} of 5.6 µg/mL.

Host cell viability trend was stay with numerous test, even we increased the concentration of the extract (Table 2). From the linear regression, the R^2 value was 0.567 (Figure 2), this indicate that no strong correlation between extract concentration and cell viability. The lowest of R^2 value in this study may due to no cytotoxic effect at the highest concentration used in this study. The CC_{50} value is 1181.1 µg/mL. We suggested for next cytotoxic assay to use butanol fraction of *Calophyllum nodosum* at concentration more than 1,000 ug/mL. The development of antiviral drug to treat DENV infection leads to sources of herbal medicines[5]. The development of small molecule anti-DENV drugs has been a slow process. To date, only four small molecule anti-DENV drugs such as chloroquine , celgosivir balapiravir and UV-4B9 already move to Phase I or Phase II clinical trials.[14] But some of them with remains unclear out come or achieved the required safety profile, but did not reduc e viral load as expected,[15,16] Furthermore, the clinical trial of the α-glucosidase inhibitor was terminated at Phase I.[14] Pre-clinical and clinical research into anti-DENV drugs is still underway, and many lessons can be learned from the previous studies. In future, we are bound to overcome the challenges, and expect our ongoing work to yield a potent anti-DENV therapy.

In this study, we used DENV-2 NGC. The promising anti-DENV drugs are anticipated to inhibit all serotypes of DENV, in the next study we will use all serotype of DENV. The antiviral drug to DENV remain challenges. The inhibition of all serotypes, as well as antibody dependent enhancement phenomenon observed during DENV infection, complicates the investigation of anti-Dengue drugs such as if a patient was re-infected by a heterotypic virus, the antibodies created previously would become severe. Moreover, in laboratory testing, the limited availability of animal models has hampered of antiviral drug to DENV development.

The value of the selectivity index of Butanol fraction of *Calophyllum nodosum* was 210.9. This value was really high compare with other study such as *Psidium guajava* and *Carica papaya* showed SI value of 21.28 and 37.25 respectively.[17] It can be concluded that the butanol fraction of *Calophyllum nodosum* has a strong antiviral effect to DENV with low cytotoxic effects. Further study needed to determine by which mechanism butanol fraction of *Calophyllum nodosum* inhibit DENV replication.

5 CONCLUSION

From this study, we concluded that $IC_{50,}$ $CC_{50\ and}$ SI value was 5.6 µg/mL, 1181.1 µg/mL, and 210.9. Butanol fraction of *Calophyllum nodosum* had strong antiviral effect against DENV-2 with low cytotoxic effect. Furthermore, Butanol fraction of *Calophyllum nodosum* can be a candidate of antiviral drug in future.

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