

Antiviral Activity of *Cynometra ramiflora* Linn Leaves Extract Against Replication of Dengue Virus Serotype 2 on Huh 7.5 Cell In Vitro

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Abstract: Dengue hemorrhagic fever (DHF) remains a major world health problem particularly in Indonesia caused by dengue virus (DENV) infection. Until now, there is no specific antiviral therapy for DENV and the treatment for its infection is still supportive. The extract of *Cynometra ramiflora* Linn leaves known for having potencies such as bactericide, analgesic, antiviral, anti-inflammation and anti-allergy. The objective of this study is to assess antiviral activity of *C. ramiflora* Linn leaves extract against DENV-2 in-vitro. The potency of *C. ramiflora* Linn leaves extract at concentration of 1.25; 2.5; 5; 10 and 20 µg/ml towards DENV was performed on Huh 7.5 cell infected by DENV-2 with moi of 0.5. Each treatment was repeated 6 times compared with DMSO treated and infected DENV-2 cells as control. Inhibition rate of the extract against DENV replication was measured using foci-forming immunoassay. Statistically, administration of *C. ramiflora* Linn leaves extract at 1,25 ; 2,5 ;5 ; 10 and 20 µg/ml resulting 36,06 %, 45,96 %, 47,35%, 55,94%, 62,70% inhibition towards DENV-2 respectively, with significant value (p < 0,05). This result showed that the extract of *C. ramiflora* Linn leaves has potency as anti-dengue.

1 INTRODUCTION

Dengue virus (DENV) infection as formed in Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) are the common infectious diseases mainly on tropical and subtropical area, Pacific Islands, South and Central America, North Australia nor Africa (Chan *et al*, 2009). Moektiwardoyo (2014) reported there is an intention to raise the number of dengue cases in Indonesia each year as the consequences of global warming. Since 1968 until 2009, WHO recorded Indonesia as a country with the highest dengue cases in South East Asia region (Indonesia Ministry of Health, 2010). In 2009 it self in Indonesia there are 77.489 cases of dengue infection with 585 fatality (Moektiwardoyo *et al*, 2014; Indonesia Ministry of Health, 2010).

DENV belong to Flaviviridae family, divided into 4 serotypes; DENV-1, DENV-2, DENV-3 and DENV-4 which each serotype was grouped into several genotypes. DENV genome composed of 11 kbp nucleotides, encoding 3 structural (prM, C and E

and 7 nonstructural (NS1, 2A, 2B, 3, 4A, 4B and 5) proteins. DENV is transmitted through *Aedes albopictus* and *Aedes aegypti* mosquito as vector (Castro *et al*, 2015).

Several efforts are conducted against DENV infection, such as vaccine development and vector control. Although dengue vaccine is now limitedly available, long term efficacy of this vaccine is remain unclear. Until now, vector control was proven can not be performed effectively nor giving a direct impact to reduce DENV infection. Not like other viral infection such as Human Immunodeficiency Virus (HIV), influenza or Hepatitis viruses which have anti-viral therapy, exact anti-DENV treatment is not available until now and referred to high burden of DENV infection this anti-DENV is urgently needed. Balancing haemostatic status is the only cure available to overcome DENV infection in patients.

Despite all of those efforts against DENV infection, several research to find a novel anti dengue is also progressing. Several plants as natural resources found to have activity against DENV. Tang

et al (2012) reported a methanol extract from *Momordica charantia* leaves and leaves extract of *Ocimum sanctum* was able to inhibit DENV-1.

Other plant that also potential to be an antiviral is *Cynometra ramiflora* Linn. Leaves of *C. ramiflora* Linn was proven to have an antiviral capability and antiseptic properties and widely applied for skin diseases, such as scabies nor leprae infection (Quattrocchi U *et al*, 2012). Several chemical compounds were found in *C. ramiflora* Linn such as flavonoids, tannin, alkaloid, phenolic and saponin (Paguigan *et al*, 2014). Saponin was reported for having a pharmacology activity such as bactericidal, antiviral, cytotoxic, analgetic, anti-inflammatory, anti-cancer and anti-allergy. Meanwhile flavonoid was found to hegative effect against prostaglandin as anti-analgetic agent (Afialus *et al*, 2013). Nevertheless, *Cynometra ramiflora* Linn is widely distributed in India, Southeast Asia and Australia (Globinmed, 2015, Hafidh *et al.*, 2009).

With its promising properties as anti-DENV and its abundancy worldwide, we assessed several concentration of *C. ramiflora* Linn leaves extract against DENV-2 in-vitro using Huh7.5 cell line. As our result show that this extract have a good potency as anti-DENV infection.

2 MATERIAL AND METHODS

2.1 Cell and Viruses

Huh7.5 hepatocarcinoma cell line was grown in MEM containing 10% FBS and 1% of antimycotic-antibiotic on 5% CO₂ at 37°C. DENV-2 NGC strain was propagated in Huh7.5 cell line and viral titer was measured with immunostaining formed as FFU/mL (focus forming unit).

C. ramiflora Linn leaves extract was diluted in Dimethyl sulfoxide (DMSO) as prepared from Indonesian Institute of Sciences (LIPI).

2.2 Antiviral Assay

A mixture of 1.25; 2.5; 5; 10 and 20 µg/ml *C. ramiflora* Linn leaves extract was prepared using 2% of FBS MEM. Thus, extract mixture was applied to a confluent Huh7.5 culture cell in 96 well plate followed by addition of DENV-2 with m.o.i (multiplicity of infection) value 0.5. Cells was then incubate on 5% CO₂ at 37°C for 1 hour.

After 1 hour of incubation a semi-overlaid media (MEM contain of 2% FBS and 0.5%

methylcellulose) was applied. Cells were then incubated on 5% CO₂ at 37°C for 48 hours.

Groups of Huh7.5 cells with DMSO and DENV-2 infected were included on each experiment as negative and positive control. Each group was performed in 6 repetitive well.

2.3 Focus Forming Immunoassay

After incubation, supernatant was discarded and cells were washed with excess PBS. A solution of 3.7% formaldehyde was added in to the cells and incubated for 15 minutes. After incubation, formaldehyde solution was discarded and cells were washed vigorously with excess PBS.

Cells were then permeabilized with 0.5% Triton-x solution for 15 minutes followed by 1:500 dengue patient sera (confirmed with RT-PCR) in 1% skim milk-PBS solution. This mixture was then incubate for 1 h RT.

After incubation, antibody was discarded and cells were then washed 3 times with excess PBS. A solution of 1:500 rabbit anti-human IgG peroxidase conjugated antibody was added in 1% skim milk-PBS followed further incubation fr 1 h RT.

After incubation, antibody was discarded and cells were then washed 3 times with excess PBS. A solution of Diaminobenzidine (DAB) substrate was then added in to the cells followed by incubation for 15 minutes at RT.

Stained group of cell will appear as brown color under microscope represent as one viral foci. Number of foci was calculated and statistical analysis were performed compared to positive control.

3 RESULT AND DISCUSSION

Our research show the potency of *C. ramiflora* Linn leaves extract against DENV-2 in-vitro using culture of Huh7.5 cell line. As we know DENV is an arbovirus with cell tropism thus the use of Huh7.5 hepatocarcinoma cell line is a suitable cell line to be used in dengue study (Blight *et al*, 2002). Antiviral potency from *C. ramiflora* Linn leaves extract against DENV-2 was ranged from 36.06%, 45.96%, 47.35%, 55.94% and 62.7% for each concentration of 1.25; 2.5; 5; 10 and 20 µg/ml respectively. As depicted from figure 1, significant DENV-2 inhibition compare to positive control was obtained in p value < 0.05 for all concentration tested.

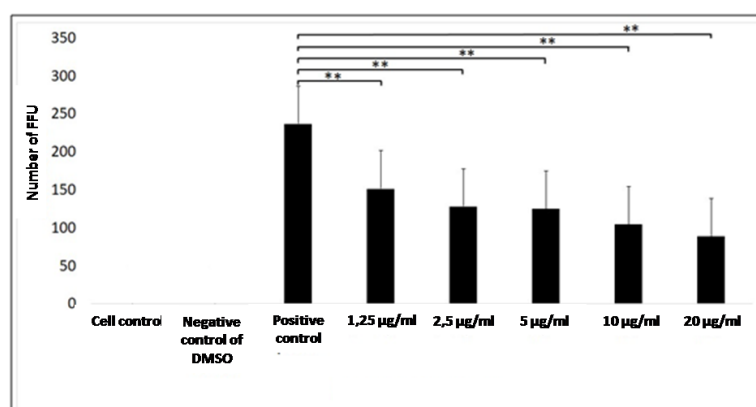


Figure 1. Number of average FFU for each concentration of *C. ramiflora* Linn leaves extract against DENV-2 represent with standard error of mean (SEM) and ** $P \leq 0,001$.

Based on phytochemical analysis, there are several chemical compounds can be found in *C. ramiflora* Linn leaves extract such as flavonoid, tannin, alkaloids phenolic and saponin (Paguigan *et al.*, 2014).

Lee *et al.* (2012) reported that saponin has the ability to inhibit Hepatitis-C virus replication through up-regulation of cytokine signal-2. We are assuming that saponin compound can also inhibit DENV replication through similar mechanism towards Hep-C virus, since both viruses belong to Flaviviridae genera. Meanwhile triterpenoid saponin, derivate of saponin, which also found in *C. ramiflora* Linn could also inhibit Herpes simplex virus (Simos *et al.*, 1999).

Flavonoid compound from *Carica papaya* can inhibit DENV enzyme of NS2B-NS3 protease which required in viral assembly which those proteins also a good target for viral inhibition (Sethivel *et al.*, 2013). Further bioinformatic analysis shown that flavonoid has high energetical bind against receptor binding site of NS2B-NS3 DENV protein. Other flavonoid deivate, quarcetin and fisetin also show DENV-2 inhibition (Zandi *et al.*, 2011).

Other compound like tannin was proved to be able to inhibit HIV-1 and influenza virus through decreasing viral peptide synthesis. Alkaloids compound such as 33 isoquinoline have antibacterial, antifungal and antiviral such as herpes simplex virus (HSV) and parainfluenza virus on varied concentration tested (Orhana *et al.*, 2007). As for phenolic compound, Ara *et al.* (2011) shown that this compound has anti replication DENV-2 activity with IC_{50} of $362,68 \pm 0,04 \mu\text{g/ml}$ and SI of 2,75.

Our result show there are DENV inhibition in the addition of *C. ramiflora* Linn leaves extract on cell

culture, although on which DENV replication steps does the inhibition appear is still unknown. Comparing similar study was performed by Meutia *et al.*, 2017 which also testing the *C. ramiflora* Linn leaves extract against DENV2 in different mtehod shows that direct and indirect measurement DENV titer may give different result of inhibition value. On the other hand, the existance of the extract during cell incubation and extract removal just after viral infection can produce different value of DENV inhibition. Moreover form this both studies, in line result was shown which *C. ramiflora* Linn leaves extract have consistent ability to inhibit DENV replication in vitro.

4 CONCLUSION

Our study shows that *C. ramiflora* Linn leaves extract is having significant anti DENV activity.

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