

Antiviral Effect of *Pterocarpus indicus* Willd Leaves Extract Against Replication of Dengue Virus (DENV) *In Vitro*

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ABSTRACT

Dengue hemorrhagic fever (DHF) is major public health problem in tropical and subtropical areas of the world with lack of approved vaccines and effective antiviral therapies. With no current treatment for illness attributed to dengue virus (DENV) infection other than supportive care, therapeutic strategies that use natural extract was developed. Indonesia have many plants that potential for antiviral drugs such as *Pterocarpus indicus* Willd (*P. indicus*). The objective of this study was to determine the effect of *P. indicus* to inhibit DENV replication. We used a well-differentiated hepatocytes-derived cellular carcinoma cell line (Huh-7 it-1 cells) to determine and select antiviral activity. The toxicity effects were determined by MTT assay. Then, the suppression of DENV replication was determined by Focus assay. Dengue infected cells with DMSO were used as control. We found that crude extract (Pi), hexane (Pi.1) and ethyl acetate (Pi.2) extract showed strong inhibition with high selectivity index (SI) of 1,392; 285.36 and 168.56 respectively. Sub fraction of Pi.1 and Pi.2 still showed strong inhibition with high SI. Further sub-sub fraction of Pi.2 such as Pi.2.12 and Pi.2.12.1 still showed inhibition of DENV replication but there was reduction of SI value. The mechanism experiment of Pi.2.12, we found that Pi 2.12 more profound to inhibit in the post infection stage that entry or pre-infection. We conclude that the sub-fraction of Pi.2.12 has potential antiviral activity against DV infection in vitro. Further studies are still needed to investigate the pure compound of Pi.2.12 that inhibit and have advantages in the future as alternative for treatment of DENV infection.

Keywords: Bioactivity, bioactive compounds, effectivity, *Neesia altissima* Bl., *Streptomyces* sp.

INTRODUCTION

Increasing in mobilization, global warming, lack of vaccine and specific treatment, DENV (DENV) become a major public health problem in tropical and subtropical areas of the world. In Indonesia, the number of reported cases started to rise in 2004 and reached a peak between 2007 and 2009. In 2015 incidence rate of DENV infection in Indonesia was 126,675 cases of DHF with 1,229 people dead [1]. Even though its high incidence, the challenges to find out antiviral against DENV have

not been success full yet until today because there is limited understanding of how the pathogens typically behaves and how the virus interacts with the immune system. Thus, no effective vaccines or therapeutics are currently available for prevention or treatment of DENV infection. Antiviral against DENV have been under development since the 1940s. Despite current efforts to control dengue, based primarily on vector control and case management, the burden and costs of the disease remain considerable.

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DENV causes a wide variety of symptoms in people with ranges from dengue fever (DF), an acute, self-limiting febrile illness, to the more severe and life-threatening dengue hemorrhagic fever and shock syndrome (DHF/DSS) [2]. Better management of the dengue needs balanced approach involving various aspects like disease prevention, cure/treatment, and the vector control, simultaneously [3]. Despite an annual number of infections approaching 50 million worldwide and a rise in disease severity, the effective anti-dengue treatment and approved vaccines remain a challenge [2]. Considering the described problems above, drug discovery research for dengue is of great importance.

DENV genome is composed of approximately 10,600 nucleotides, single stranded positive sense RNA. It contains a single open reading frame that is flanked by two un-translated regions; 5' and 3' un-translated region (UTR). There are four distinct serotypes of DENV known as DENV-1, DENV-2, DENV-3, DENV-4 [4]. The treatment of DENV was developed based on the characteristics of DENV. An antiviral drug that targets a common host pathway could provide an advantage to the issues faced by therapies targeting viral enzymes or mechanisms, such as viral heterogeneity and the emergence of drug resistant mutants. In vitro study showed that there was inhibition of 4 serotype of DENV replication in Huh 7.5 cell line using seaweed extract [5]. Statistical analysis showed an eight seaweed extracts such as Phaeophyta: *Canistrocarpus cervicornis*, *Padina gymnospora*; Rhodophyta: *Palisada perforate*; Chlorophyta: *Caulerpa racemosa* were able to reduce DENV infection cycle, such as binding or internalization of at least one serotype tested [5].

The development of specific treatment has been moved to *in vivo* study. Perry *et al.* 2013 described an amino sugar drug UV-4 to protect lethal DENV challenge in AG129 mice [6] with lacking the type I and II interferon receptors [7]. They found that administration of UV-4 reduced mortality, as well as viremia and viral RNA in key tissues, and cytokine storm [7].

Indonesia have many plants that potential for antiviral drugs such as *Pterocarpus indicus* Willd (*P. indicus*). *P. indicus* belongs to family Leguminosae and has been used as green plant all over big city in Indonesia. In Indonesia, this plant is recognized as *Sono kembang* and *Cendana Merah* [8]. Other researcher has identified and evaluated effect of *P. indicus* to the inhibition of bacteria growth. The leaves, root and stem barks of *P. indicus* were successively partitioned with petrol, dichloromethane, ethyl acetate, butanol, and methanol.

All the fractions exhibited a wide spectrum of antibacterial activity. The activity was more pronounced in the butanol and methanol fractions [9]. The bark of *P. indicus* becomes a kind of red crystal after some hours of exposure to air, which is used as an astringent and against other diseases [10]. The structural analysis shows that the crystal is a macromolecular compound of tannic condensation and glucoside [10].

We examined the antiviral effect of *P. indicus* leaves to DENV-1 strain IDS 11/10 in Huh-7 it-1 cells *in vitro*. DENV-1 strain IDS 11/10 was isolated from DF patient in the community of Jakarta, Indonesia in 2010. The purpose of this study was to determine the dose and mechanism of inhibition of *P. indicus* that can be used to inhibit DENV replication.

MATERIALS AND METHODS

Collection of plants and preparation of plant extract

P. indicus leaves were collected from Research Center for Chemistry, Indonesian Institute of Science (LIPI), Indonesia as previous study description [11]. The botanical identities of each plant were determined and authenticated by the Botanical in Research Center for Biology LIPI. After the taxonomy identification, the plants were washed and cleaned prior to air drying at room temperature. The extract was initially partitioned with n-hexane, ethyl acetate, butanol, and water. The antiviral candidate from ethyl acetate fraction was subjected to silica gel column chromatography with diluents of n-hexane and ethyl acetate with equal volume. The major fraction was monitored in TLC and all fractions were tested for antiviral to DENV. Furthermore, the candidate was purified by Sepadex LH-20 column using 50%, 60% and 80% of methanol.

Preparation of plant extracts for in vitro testing

Extract, fractions and sub fraction were diluted at concentration of 100 mg/ml in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) as a stock solution. Centrifugation was done to remove insoluble material. The stock solution was diluted with culture medium to serial dilution for the assays.

Maintenance of Huh-7 cells

Dulbecco's Modified Eagle's Medium (DMEM) High glucose cat no 11965-092 (GIBCO, UK) was used for maintenance and antiviral assay on Huh7it-1 cells. The cryo-preserved Huh7it-1 cells were rapidly thawed at 37°C. Cells were transferred carefully to 25 cm² tissue culture flasks (Corning, USA) containing 5 mL of

DMEM with 10% of Fetal Bovine Serum (FBS) (GIBCO). Cells were then incubated at 37°C with 5% CO₂ for 4-5 days until confluent.

Preparation of DENV

We used DENV isolated from patients IDS 11/10 in Jakarta in 2010. C6/36 cell line was used to isolate DENV at Department of Microbiology, Faculty of Medicine, University of Indonesia [12]. The presence of DENV in supernatant of C6/36 cell was checked by HA [13] and plaque assay [12]. When the supernatant cell showed positive by HA or plaque, we propagated DENV in T-75 flask. A monolayer of C6/36 cell in T-75 flasks were infected with DENV at an moi 0.5 FFU/cell and incubate at 28°C for 7 days. During the time of virus propagation, the FBS concentration of the cell culture medium was reduced to 2%. Supernatant was harvested and centrifuged at 900 g for 5 minutes. Subsequently, filtered using a syringe driven 0.22 µm (Millipore, Co. Bedford MA USA). Culture supernatant was subsequently stored at -80°C and checked for the titer of DENV by plaque assay [12] or focus forming assay on Vero cell [14]. Supernatant was then collected and stored at -80°C. These were repeated for several times until adequate virus stock was collected. In this study we used DENV-1 at 5 to 10 passages.

DENV strain IDS 11/10 confirmed by Reverse Transcription Polymerase Chain Reaction [15] and sequencing. The DENV obtained was confirmed to be DENV-1 (data not shown).

Determination of cytotoxicity (CC₅₀)

In vitro cytotoxicity (CC₅₀) was determine by viability of Huh-7 cells after treated with natural extract. The viability assay was initiated by seeding 2 × 10⁴ cells into 96 well flat-bottom tissue culture plates (Corning, USA). A blank control (medium only) and cell control (cells only) were also plated. After 24 hours, the cells were treated with diluted stock of extracts at the concentrations ranging from 0.1 to 80 µg/mL, incubated at 37°C, 5% CO₂. After 48 hours, 20 µL 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. The absorbance reading of each well was measured using micro plate reader at 490 nm. The percentage of cell viability and toxicity was further determined based on the absorbance readings. We calculated the theoretical percentage toxicity of the samples by dividing the mean blanked sample ODs by the mean blanked control ODs for each sample.

We used percent viability to represent cytotoxic effect. The viability of the cells was determined by this following equation:

$$\text{Cell viability} = \frac{\bar{x} \text{ OD of Sample}}{\bar{x} \text{ OD of DMSO}} \times 100\%$$

The percentage viability cell, we used to determine CC₅₀. The CC₅₀ was obtained from nonlinear regression analysis of concentration-effect curves by the Graph and represented means ± standard deviation experiments.

Determination of antiviral activity (IC₅₀)

A total of 2 × 10⁴ cells/well were seeded into 96 well plate and incubated at 37°C with 5% CO₂. After 24 hours, the cells were infected with DENV-1 at a m.o.i. of 1 FFU/cell which contained various concentration of natural extract. Those concentration were 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 0.25 µg/mL and 0.125 µg/mL. After 2 hours infection, added 100 µL of DMEM +2% FBS contained various concentration of natural extract. Plates were further incubated at 37°C for 3 days. Then we harvested to determine antiviral activity by counting the virus titer with focus assay [14] with slight modification. Briefly, the mixtures of 10-fold serial dilution of viral supernatant was inoculated onto Vero cell monolayer in duplicate wells. Absorption was carried out at 35°C in 5% CO₂ for 2 hours with agitation at 30 minutes interval. Methylcellulose 1.5% overlay medium was added and infected Vero cells were incubated at 35°C in 5% CO₂ for 3 days. The infected Vero cells were fix with 10% formaldehyde in PBS and incubated at room temperature for 1 hour. The cells were washed with PBS 3 times. As much as 100 µL /well of 1% of Nonidet P40 were added to each well and incubated at room temperature for 30 minutes. Then, the cell was blocked with 5% skim milk in PBS and incubated at room temperature for 1 hour. After washed, the cell was added with 1/1000 of human IgG anti dengue and incubated at room temperature for 1 hour. We used 1/1000 antihuman IgG labeled HRP as a secondary antibody. After washed, we added substrate and observed infected cell with brown color. The result from focus assay was used to determine IC₅₀ with following equation:

$$\text{Inhibition (\%)} = \frac{\bar{x} \text{ titer DMSO} - \bar{x} \text{ titer treatment}}{\text{Average titer DMSO}} \times 100\%$$

Based on percentage inhibition, we able to determine IC₅₀. The IC₅₀ was obtained from nonlinear regression analysis of concentration-effect curves by the graph

Table 1. Results of IC_{50} , CC_{50} and Selectivity Index (SI) of crude extract of *P. indicus*

Component	Code	CC_{50} ($\mu\text{g/mL}$)	IC_{50} ($\mu\text{g/mL}$)	Selectivity Index (SI)
Crude Extract	Pi	174.31	< 0.125	>1,392.00
Hexane	Pi.1	35.67	< 0.125	> 285.36
Ethyl acetate	Pi.2	21.07	< 0.125	>168.56
Butanol	Pi.3	11.75	15.68	0.75
Water	Pi.4	21.37	20.76	1.03

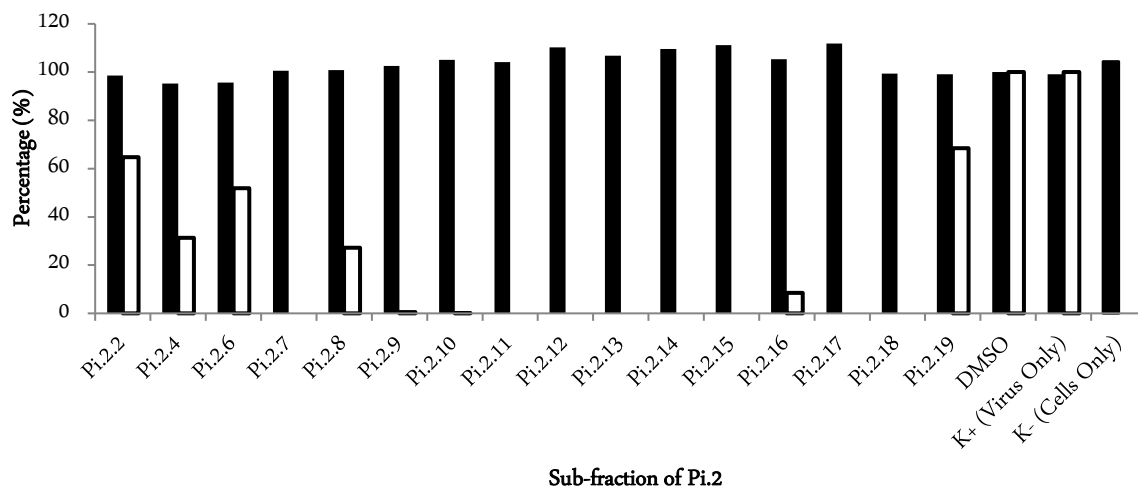


Figure 1. The percentage of viability (black) and infectivity (white) of sub-fraction of Pi.2 with single dose of 30 $\mu\text{g/mL}$

and represented the means \pm standard deviation experiments.

Statistically analysis

The normality of percentage of viability and inhibition of infection was analyzed by Shapiro Wilk. Statistical difference among treatments and control was analyzed using ANOVA at p value less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

IC₅₀, CC₅₀ and SI of P. indicus

The crude extract of *P. indicus* leaves showed concentration to inhibit 50% of DENV replication (IC_{50}) of < 0.125 $\mu\text{g/mL}$. The hexane and Ethyl acetate fraction of *P. indicus* leaves showed very strong inhibition of DENV replication with IC_{50} of < 0.125 $\mu\text{g/mL}$. In the other hand, butanol and water fraction of *P. indicus* leaves indicated low inhibition of DENV replication with IC_{50} of 15.68 $\mu\text{g/mL}$ and 20.76 $\mu\text{g/mL}$, respectively (Table 1).

Base on viability assay, the crude extract of *P. indicus* leaves showed low cytotoxic with concentration to destroy 50% of cells (CC_{50}) of 174.31 $\mu\text{g/mL}$. The hexane and ethyl acetate fraction of *P. indicus* leaves showed CC_{50} of 35.67 $\mu\text{g/mL}$ and 21.07 $\mu\text{g/mL}$. Butanol and water fraction of *P. indicus* leaves gave almost similar

result of the CC_{50} : 11.75 $\mu\text{g/mL}$ and 21.37 $\mu\text{g/mL}$, respectively (Table 1).

The selectivity index (SI) was formulated by dividing CC_{50} with IC_{50} . The SI of crude extract of *P. indicus* showed high SI value of 1,392. The hexane and ethyl acetate fraction of *P. indicus* leaves showed SI value of 285.36 and 168.56. Butanol and water fraction of *P. indicus* leaves gave SI value of 0.75 and 1.03 respectively (Table 1).

IC₅₀, CC₅₀ and SI of Sub-fraction of P. indicus

Based on the SI value and chemical characteristic, we continued sub-fraction of Pi .2 not Pi.1. We got 16 sub-fractions. We screened those sub-fractions only with single dose of 30 $\mu\text{g/mL}$. The percentage of viability and infectivity was shown in Figure 1. Pi.2.9 and Pi.2.10 showed high viability with 100% of inhibition DENV infection. Similar result was shown after treated with Pi.2.11, Pi.2.12; Pi.2.13; Pi.2.14 and Pi.2.15. The high viability and without any infection also were shown after treated with Pi.2.17 and Pi.2.18

IC₅₀, CC₅₀ and SI of sub-fraction of P. indicus

We continued to determine IC_{50} , CC_{50} , and Selectivity Index (SI) of sub-fraction of *P. indicus* in Huh-7

Table 2. IC_{50} , CC_{50} and Selectivity Index (SI) of sub fraction of *P. indicus*

Sample	CC_{50} ($\mu\text{g/mL}$)	IC_{50} ($\mu\text{g/mL}$)	SI
Pi.2.9	424.6	26.8	15.84
Pi.2.10	228.9	26.2	8.72
Pi.2.11	27.5	10.3	2.65
Pi.2.12	72.4	3.8	19.05
Pi.2.13	401	15.9	25.22
Pi.2.14	60	3.03	19.8
Pi.2.15	295.4	15.1	19.56
Pi.2.17	217	24.8	8.75
Pi.2.18	1288	40	32.2
Pi.2.12.1	1174	48.6	24.16

Table 3. The percentage of inhibition of DENV infection at entry stage, pre, post and pre-post infection of DENV by Pi.2.12. at concentration of 10 $\mu\text{g/mL}$

Treatment	Inhibition of infectivity	
	Average (%)	SD
Entry	24.23	6.11
Pre	63.65	2.88
Post	90.19	1.15
Pre- and post-	100	0

cells which high inhibition and low cytotoxic (Table 1). We found that Pi.2.18 has the strongest antiviral activity compare with another sub-fraction. Some of *P. indicus* sub-fractions such as Pi.2.12, Pi.2.13, Pi.2.14, and Pi.2.15.1 were very low cytotoxic effect to the Huh-7 cells.

IC₅₀, CC₅₀ and SI of sub-fraction Pi 2.12.1

Due to chemical characteristic, strong inhibition and practical to isolate the pure compound, we continued to sub fraction on Pi 2.12. From the sub fraction we found fraction that we code it as Pi 2.12.1. We evaluated the effect of Pi.2.12.1 to the replication of DENV using focus assay. Pi 2.12.1 showed IC_{50} , and CC_{50} of 1,174 $\mu\text{g/mL}$ and 48.6 $\mu\text{g/mL}$ respectively (Table 2). The selectivity index of Pi 2.12.1 was 24.16.

The mechanism of inhibition (pre- and post-inhibition)

Beside continuing sub fraction of Pi 2.12, we also determined the inhibition mechanism by which Pi.2.12 inhibit DENV replication. We used single dose of 10 $\mu\text{g/mL}$ (above IC_{50} value) of Pi.2.12 (Table 2). We found that treatment at entry step, that mean addition

of Pi.2.12 to block DENV receptor at Huh7 it-1 showed lowest inhibition (24.23%) of DENV replication. Addition of Pi.2.12 at DENV infected Huh7 it-1 cell (post infection) showed strong inhibition (Table 3). Similar result was found in pre- and post-inhibition. Pre- and post-inhibition mechanism was strongest compare all of treatments with 100% inhibition (Table 3).

The important properties of medicinal plants since it has multiple targets, minor side-effects, low resistance due to selective pressure of infective agents and low cost [16, 17, 18, 19]. Recently, with high technology in chemistry and particularly of extraction methods, active principles of such medicinal plants have been isolated and studied. This result became templates for the synthesis of analogue drugs and thus heralded the advent of synthetic drugs [20]. Bark of *P. indicus* showed carcinostatic effect on ascetic mice with Erlich carcinoma [21]. Various extracts of leaves, root, and stem barks of *P. indicus* were studied for antibacterial activity. All fractions exhibited a wide spectrum of antibacterial activity, more pronounced in the butanol and methanol fractions [9].

In a screening for potential therapeutic agents against DENV, we evaluated leaves extract of *P. indicus* from Indonesia. The crude extract of *P. indicus* (Pi) have antiviral effect to DENV with high selectivity Index. Hexan (Pi1) and ethyl acetate (Pi2) of *P. indicus* leaves extract exerted a strong inhibitory effect against DENV with selectivity index of 285.36 and 168.56, respectively. Other study found that ethanol extract of *P. indicus* leaves showed strong inhibition on *Staphylococcus aureus* growth but low inhibition in *Streptococcus pyogenes* and *Escherichia coli* [22]. Chloroform extract did not show any inhibition in bacteria growth [22]. Crude extract showed strong inhibition in contrast sub-sub fraction of Pi.2 with code of Pi 2.12.1 even low cytotoxic but did not show strong inhibition even Pi.2.12 showed strong inhibition. This result indicated that purification has led to loss of activity, which suggests that components of the crude extract acted either in synergy or additively to produce the antiviral activity. Situations where purification has led to loss of activity have been reported. Even not in all cases, fractionation of *Tamarindus indica* led to loss of activity to *E. coli* [23]. In the study of antifungal activity, demonstrated the lost activity in some medicinal plants after the separation of the plant extracts into precipitant and supernatant [24].

Understanding of the relevant events during early DENV infection has been improved the specific inhibitors from natural extract. DENV is a positive-strand

RNA virus and contain three structural proteins: the capsid (C), the membrane (M), and the envelope (E) protein [25]. In the infected cell, the M protein is produced as a precursor protein called prM, which its function as a chaperone during the folding and assembly of the E protein [26, 27]. The initial step in the life cycle of DENV is attachment of the virus to a cellular receptor. DENV has been proposed to bind to the glycosaminoglycan heparan sulfate, which is expressed on many cell types [28, 29, 30, 31]. Study *in vitro* investigated the cell entry characteristics of DENV-2 strain S1 on mosquito, BHK-15, and BS-C-1 cells [32]. It was observed the particles that did bind to the cells showed different types of transport behavior leading to membrane fusion in both the periphery and perinuclear regions of the cell. Membrane fusion was observed in 1 out of 6 bound virus particles, indicating that a substantial fraction of the virus has the capacity to fuse. Fusion occurs exclusively from within acidic endosomes. In the experiment to determine DENV inhibition mechanism by which we used a single dose of 10 µg/mL (above IC₅₀ value) of Pi.2.12 (Table 2). We found that treatment at entry step, that mean addition of Pi.2.12 to block DENV receptor at Huh7 it-1 showed lowest inhibition (24.23%) of DENV replication. Addition of Pi.2.12 at DENV infected Huh7 it-1 cell (post infection) showed strong inhibition (Table 3). Similar result was found in pre- and post-inhibition. Pre- and post-inhibition mechanism was strongest compare all of treatments with 100% inhibition (Table 3).

Previous study has investigated the parameter specific standardization shown organoleptic extract *P. indicus* (thick, blackish brown green color, weak and do not have special odor, and bitter tasted), with the content of water-soluble compounds (22.88 ± 0.41 to 24.44 ± 3.98), and ethanol-soluble (13.62 ± 1.206 to 15.37 ± 0.72), and total flavonoid levels (3.88% - 4.02%). The result of non-specific parameters test shown that the content of water (13.84 ± 3.59 to 20.60 ± 2.13), drying shrinkage (15.85 ± 1.58 to 33.37 ± 2.84), ash extract (5.51 ± 0.57 to 7.63 ± 1.53), insoluble ash in acid (0.06 ± 0.04 to 1.49 ± 0.25), and specific gravity (1.01 ± 0.002 to 1.02 ± 0.01). The microbial contamination test resulted (60* to 130* colonies/g), while the test of mold yeasts contamination (0 to 45* colonies/g × 10⁻³ mg/kg), material lead (0.002 to 0.003 mg/kg), and cadmium (0.000011 to 0.000021 mg/kg), as well as arsenic (0.208 × 10⁻³ mg/kg - 0.956 [11]).

P. indicus was a good source of traditional medicine and it provides a noteworthy basis in pharmaceutical biology for the development or formulation of new drugs

and future clinical uses to combat DENV infection. Further studies needed to determine mechanism of inhibition of infection using crude extract of *P. indicus*. Since it has antiviral effect to DENV with high selectivity Index.

CONCLUSION

Crude extract (Pi), hexane (Pi.1) and ethyl acetate (Pi.2) extract showed strong inhibition with high selectivity index (SI) of 1,392; 285.36 and 168.56 respectively. The mechanism action of sub- sub fraction Pi.2, showed that Pi 2.12 more profound to inhibit in the post infection stage that entry or pre-infection. Sub-sub fraction of Pi.2.12 has potential antiviral activity against DV infection *in vitro*.

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