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The Potency of *Piper betle* Leave Extract as Antiviral Drug to Dengue Virus In Vitro

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ABSTRACT

Up to now, there is no specific medicine known to be effective in treating dengue virus (DENV) infection, there is only supportive treatment geared toward its symptoms. It is known that herbal extracts possess a measure of effectivity against DENV. Piper betle, or sirih, is one of the most common medicinal plant used by Indonesian citizen. Therefore, an in-depth study regarding the effectivity of Piper betle leave extract towards DENV becomes an important topic. Huh 7it-1 cell was cultured on plat 96 and 48 well plate to examine toxicity and effectivity, respectively. Then, the cells were infected with DENV which has been given Piper betle leave extract at various concentration such as 160ug/mL, 80ug/mL, 40ug/mL, 20ug/mL, 10ug/mL, and 5ug/mL. After incubation for 48 hours, supernatant and cell were harvested to conduct focus and MTT assay respectively. From this study we found that Piper betle has an IC_{50}, CC_{50} and Selectivity Index of 17.662 µg/ml, 486.404µg/ml, and 24.3 respectively. It is indicating that Piper betle has a strong potential in becoming an alternative antiviral agent for dengue virus. Further experimental investigations are needed to determine cytotoxic and effectivity of Piper betle leave extract as an antiviral to DENV in vivo.

1. INTRODUCTION

DENV infection is a serious community health problem in the past and it still is today. Between 2004 and 2010, 390 million people are infected each year in the world. With 75% of DENV infection is concentrated in the Asia Pacific and Indonesia as the 2nd country with the highest number of DENV cases [1]. The death count of DENV is recorded at 493 cases and an incidence rate of 26,12 cases for every 100.000 people in 2017 [1]. There is a significant increase in the incidence rate of dengue infection over the past 50 years [1]. The number has increased from 0.05 cases for every 100,000 people in the year 1968, to 77.96 cases for every 100,000 people in the year 2016.2 However, compared to the increase of incidence rates, the number of CFR (Case Fatality Rate) decrease over the past 50 years [2]. During the 1960s the number of CFR is

calculated to be at 20% of the people infected with dengue [2]. While in 2016, this number experience a significant decrease, becoming 0.79% of the people infected with DENV-2. Once again, highlighting the prevalence of DENV today. Clinical manifestations of DENV infection form a broad spectrum and include uncomplicated dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [3]. Warning signs occur 3 until 7 days after the first symptom accompanied by decreasing temperature and other dangerous symptoms.3 These symptoms include persistent vomiting, severe abdominal pain, rapid breathing, fatigue, restlessness, bleeding gums, and blood found in vomit [3]. To date, there is no established specific treatment to manage DENV infected patients [4]. However, there are alternative prevention and therapies [4]. Among these alternative therapies, the use of plants such as Azadirachta indica, Hippophae rhamnoides, Carica Papaya, and Cissampelos pareira are reported to be used in India [4]. The validity of the effects of these plants on the dengue virus is still being discussed [4]. However, there are studies on the antiviral effects of medicinal plants on dengue [5]. In general, compared to synthetic drugs, medicinal plants or natural drugs are less toxic and less expensive [5]. Making the study of plants extracts effects on DENV worthwhile. On another front, there is currently DENV antivirus drug development which targets the structural proteins or non-structural proteins of the virus instead [6]. Although no DENV specific drug enters had entered clinical trials, a drug, originally used for hepatitis C, called Balapiravir is known to directly target the non-structural protein 5 (NS-5) [6]. An ideal DENV drug will have a great activity on all DENV serotypes, has a high safety profile, and dissolvable through oral since there is a lot of pediatric cases [6]. Several compounds such as previously mentioned Balapiravir along with prednisolone, chloroquine, celgosivir, and lovastatin [6]. All these compounds have failed to meet the required primary efficacy [6]. However, a step forward had been made on this front through all the failed testing [6]. It is expected that these failed results can be used to further advance DENV antivirus drug development [6].

Piper betle extracts or Piper betle extracts especially leave extract are known to have multiple medicinal properties and their widespread use as herbal medicine is well known. Their active components had been used as antimicrobial, antitumor, antioxidants, neuroprotective activity, antihelmintic, and antidiabetic activity [7]. Known active component of piper betle leaf includes chavicol, piperbetol, hydroxychavicol, chavibetol, methylpiperbetol, piperol A, and piperol [7]. However, currently, the potency of Piper betle leave extract antiviral activity on DENV is not known. Therefore, the aim of this study was to identify the potency of Piper betle leave extract as an antiviral agent of DENV through the Inhibitory Concentration (IC_{50}) and Cytotoxic Concentration (CC_{50}) assessment. In vitro study using focus assay and MTT assay were used to determine the IC_{50} and CC_{50} respectively. It was expected that this study could be used to find an antiviral drug to DENV in the future.

2. Materials and Methods

2.1 Culture medium preparation

We used high sugar DMEM containing L-Glutamin and FBS 10%, NEAA and Kanamycin 0.3% to culture Huh 7it-1 [20]. For a sterility test of the medium, we added 1 mL of medium to thioglycollate and then stored at temperature of room temperature for 24 hours.

2.2 Huh7it-1 cell preparation

Huh7it-1 cell were prepared according to previous study [20]. For antiviral assay Huh7it-1 cell were harvested and seeded in 48 well plate [20]. The cell was incubated at 37^{o}C with 5% CO_2 for 24 hours [20].

2.3 Determining of IC_{50}

Huh7it-1 cell monolayer at 48 well plate was added with, mixture of DENV at a multiplicity of infection

(MOI) of 0.5 FFU/cell and various concentration of extracts. The various concentration in this study were 160µg/mL; 80µg/mL; 40µg/mL; 20µg/mL; and 10µg/mL, along with DMSO 0.3% as the control [20]. Then, the cell was incubated at 37ºC with 5% CO_2 for 48 hours to allow DENV replication. After that supernatant of culture cells were harvested and measured the titre of DENV by focus assay.

2.4 Focus assay test

For focus assay we used Vero cells in 96 well plate. After 24 hours' incubation, supernatant of DENV treated with various concentration and DMSO were added. [20] Before addition to the cell, the supernatant was diluted 10 and 100 times. After 2 hours' incubation, cells were added with 100 µL/well of methylcellulose 0.5% and incubated for 48 hours. The cells were fixed into the plate using 200 µL/well formaldehyde 3.7%. After fixation we add Triton X-100, 100 µL/well and incubated for 30 minutes. After washing with PBS 3 times, the cell was added with 50 µL/well, 1/500 in PBS with 1% skim milk of IgG anti dengue human serum antibody and incubated for 1 hour. Antibodies were disposed of and washed with 200 µl PBS. On the second antibody, 50 µl/well, 1/1000 IgG anti human labeled Horseradish peroxidase (HRP) in PBS with 1% skim milk were added. After 1 hour of incubation, the second antibody are disposed of and washed without interval. Then, 100 µL/well of substrate containing DAB and H2O2 were added and incubated for 15 minutes. After substrate was discarded, we added distilled water and counted the brown focus under the microscope. After this, the infectivity percentage were counted using the following formula:

Infectivity Percentage (%) = (Piper betle extract FFU/Average DMSO FFU) x 100

Then, a graph was obtained using an average of infectivity percentage at each concentration. A trendline was set closest to obtain a R^2 using a linear or exponential regression.

2.5 Determining CC_{50}

We used MTT assay to determine the CC50. At 96 well plate was seeded 100 µL/well of Vero cells at concentration of $2x10^5$ cells/mL. Plate were incubated at 37ºC and 5% CO_2 for 24 hours. After incubation, the medium was disposed. A mixture of extract and medium at concentration of 80µg/mL; 40µg/mL; 20µg/mL; 10µg/mL and 5µg/mL were added. The plate was incubated at 37ºC with 5% CO2 for 48 hours. After incubation, the viability of the cells was counted using MTT assay. Medium was disposed and replaced with a 150 µl/well mixture of MTT reagent and complete medium, which were incubated at 37ºC with 5% CO_2 for 3 hours. The mixture then was disposed, after which 100 µl/well of DMSO 100% was added. Plate was read using ELISA Reader at wavelength 490 nm. The cell viability percentage are counted using the following formula:

Viability percentage (%) = (Absorbance sample/Average DMSO) x100

Then, a graph was obtained using an average of viability percentage at each concentration. A trendline was set closest to obtain a R^2 using a linear or exponential regression.

2.6 Selectivity index

Selectivity index (SI) will then be counted using the formula:

$$SI = \frac{CC50}{IC50}$$

3. Results

3.1 IC_{50} of Piper betle

We used Focus assay to determine the IC_{50} of Piper betle extract against DENV. One focus represented one DENV after treated with various extract concentration and DMSO (Table 1).

Table 1. Number of focus in cells infected with DENV treated with various concentration of extracts

Piper betle **Concentration (µg/mL)**	**Number of Focus (n)**			**Average ± SD**
160	0	1	1	0.7 ± 0.3
80	8	29	13	16.7 ± 6.7
40	51	89	51	63.7 ± 12.7
20	87	86	102	91.7 ± 5.2
10	116	226	148	163.3 ± 32.7
DMSO 0.3%	235	205	243	227.7

We found that the number of focus in the wells treated with Piper betle extract, were generally less than those treated with DMSO (Table 1). Treated with higher concentration of extract showed a decrease of focus number. The number of focus were then inserted using the formula to obtain infectivity percentage. Normality tests were done at each concentration using Shapiro-wilk. We found that treatment of 160 µg/mL and 40 µg/mL extract concentration, the data was not normal with p value of 0 for both concentrations. Therefore, those data were presented in median with minimum and maximum value. Treatment with 80 µg/mL, 20 µg/mL, 10 µg/mL, showed a p value > 0.05. Therefore, the data were presented in average with standard deviation. After treated with 160 µg/mL we found the infectivity percentage to be 0.4% (Table 2). When the concentration of extract was decreased, the infectivity percentage increased (Table 2). To determine the correlation between concentrations and infectivity percentage we used ANOVA test. Since there were abnormal data, the data were transformed before subjected to ANOVA test. The p value of ANOVA test showed there were correlation between concentration with infectivity percentage (p value < 0,01).

Table 2. Infectivity percentage of DENV after treated with Piper betle Extract and DMSO

Piper betle **Concentration (µg/mL)**	**Infectivity (%)**			**Average ±SD**
160	0.0	0.4	0.4	0.4 (0.0-0.4)*
80	3.5	12.7	5.7	7.3 ±4.8
40	22.4	39.1	22.4	22.4 (22.4 – 39.1)*
20	38.2	37.8	44.8	40.3 ±3.9

10	50.9	99.3	65.0	71.7 ±24.8
DMSO 0.3%	103.2	90.0	106.7	100.0

*Median Value (Minimum – Maximum)

Through the average infectivity percentage, a graph was made to visualized the correlation between infectivity percentage and Piper betle concentration (Figure 1). As the graph showed, there was a correlation between the two variables. To find the IC_{50}, an equation was derived from the graph with an equation of $y=91.152e^{-0.034x}$. An exponential graph was used to obtain R^2 value. From the graph, we found R^2 value 0.99626 (Figure 1). From the equation we substituted Y-axis (infectivity percentage %) to find infectivity at 50%. After calculation, the value of X-axis was found to be 17.662 which translated to IC_{50} = 17.662 µg/mL.

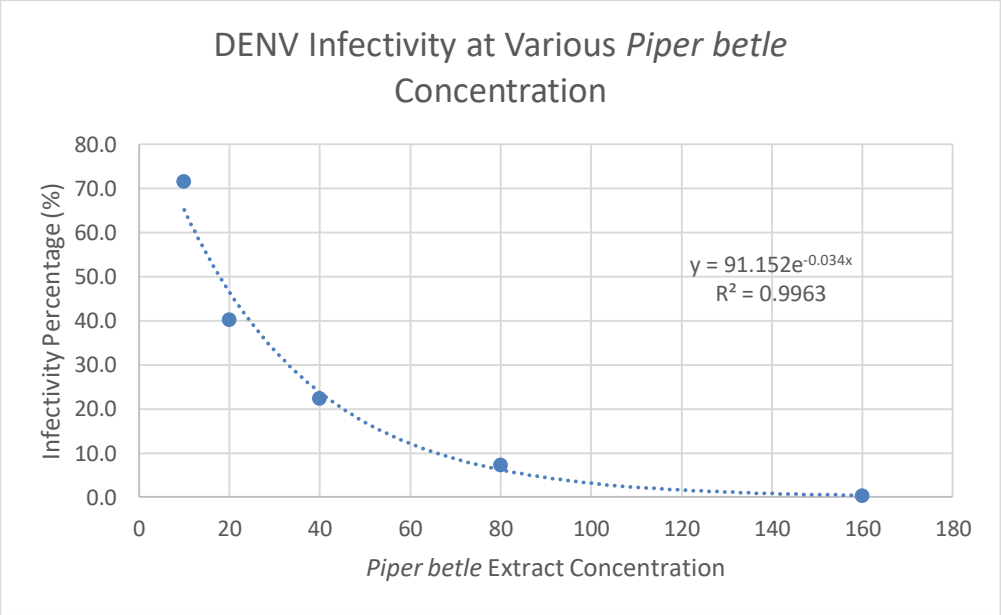

Figure 1. Correlation between DENV infectivity and Piper betle extract concentration

3.2 CC_{50} of Piper betle Extract

CC_{50} of Piper betle extract can be deduced through MTT assay. The optical density of the cell after treated with concentration of 80 µg/mL, still showed similar results with DMSO treatment (Table 3).

Table 3. Optical density of the cell after treated with Piper betle extract and DMSO

***Piper betle* Concentration (µg/mL)**	**Optical Density**			**Average ±SD**
80	0.807	0.892	0.823	0.841 ± 0.026
40	0.847	0.884	0.849	0.860 ± 0.012
20	0.857	0.877	0.916	0.883 ± 0.017

10	0.851	0.907	0.965	0.908 ± 0.033
5	0.895	0.945	0.909	0.916 ± 0.015
DMSO 0.3%	0.808	0.919	0.867	0.865

Optical density data from MTT assay (Table 3) were then inserted into the formula to obtain the viability percentage. Then, normality tests were done on each concentration using the normality test Shapiro-wilk. Normality tests showed that the data obtain from concentration 80 µg/mL to 5 µg/mL were normal and thus, the data were presented with average and standard deviation. Similar to infectivity percentage, correlation between infectivity percentage and concentrations were determined using ANOVA test. The p value of the ANOVA test was 0.157 which showed that there was no correlation between viability percentage and Piper betle concentration.

Table 4. Percentage of cell viability after treated with various concentration of Piper betle Extract or DMSO

Piper betle **Concentration (µg/mL)**	**Viability (%)**			**Average ±SD**
80	93.3	103.1	95.1	97.2 ±5.2
40	97.9	102.2	98.2	99.4 ±2.4
20	99.1	101.4	105.9	102.1 ±3.5
10	98.4	104.9	111.6	105.0 ±6.6
5	103.5	109.2	105.1	105.9 ±2.9
DMSO 0.3%	93.4	106.2	100.2	100.0

From the viability percentage, a graph was derived (Figure 2). The CC_{50} was obtained through the use of this graph. Firstly, an equation was obtained from the line of best fit which in this case was y= -0.114x + 105.45 after which, we input the value of 50 on the Y-axis (Viability percentage %). This was done to obtain the concentration at which the viability percentage was at 50%. After calculation, the X-axis (Piper betle concentration) was found to be 486.404µg/mL which means the CC_{50} = 486.404µg/mL.

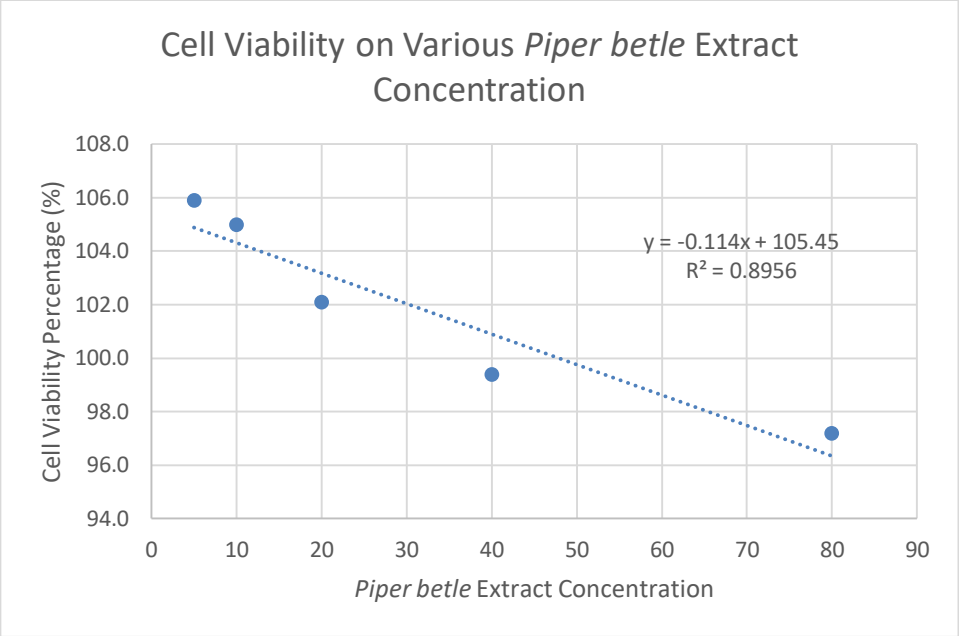

Figure 2. Correlation between cell viability and Piper betle extract concentration

3.3 Selectivity Index

Selectivity index was measured using the following equation:

$$SI = CC_{50} / IC_{50}$$
$$SI = 486.404 / 17.662$$
$$SI = 27.5$$

From the CC_{50} and IC_{50} we managed to obtain the selectivity index of Piper betle.

4. Discussion

Currently, the management of DENV leans on supporting care such as judicious fluid replacement along with close monitoring [17]. So far, no specific antiviral drug is commercially available even though there is a connection between higher viremia levels with higher chances of severe dengue [17]. DENV is a mosquito-borne arboviral disease along with chikungunya and zika [5]. However, it is concluded that DENV one of the most important of the three [5]. DENV is very prevalent in tropical and also, the sub-tropical regions of the world [5]. Along with the fact that it is very adapted to the urban areas of the city, makes the DENV epidemic very hard to combat against [5]. Antiviral drugs in developments are mainly focusing on the structural and non-structural proteins [17], [18]. Non-structural proteins 5 (NS5) as an RNA dependent RNA polymerase and NS3 as a protease are the primary target of drug development for DENV [17], [18]. Beside natural extract from herbal medicine in the development of drugs, there iare also much research focusing on the repurposing of available drugs [17], [18]. Natural extracts as a natural products are now known to bring about a positive and significant effects as an antivirus of DENV in humans or animals model [19]. Cyclohexenyl chaclone derivatives along with panduratin obtained from Boesenbergia rotunda shows an inhibitory effect to DENV-2 protease. [19] Some other plants known to have inhibitory effects towards DENV are Momordica charantia, Andrographis paniculata, and Azidarachta indica [19]. The exact mechanism action of extract may vary. [19] Some affect the DENV replication at various points [19]. Knowing where the extract acts on is important strategies in the development of antiviral therapies and applications [19]. It is confirmed that herbal extract such as Cassia alata and Cosmos caudatus leaf extract

possesses an anti-viral effect [20]. In a study, the antiviral effect of medicinal plants on in Huh7it-1 cell against DENV2 serotype of dengue virus was tested and recorded [20]. In comparison with other study, IC_{50} of Ethyl Acetate (EA) fraction of Houttuynia cordata (H cordata) Thunb was 7.50µg/ml using C6/36 and BHK-21 (baby hamster kidney) cell line [21]. This is lower than the IC_{50} of Piper betle of 17.662 µg/ml. However, the IC_{50} of Piper betle was lower than the IC_{50} of Taraxacum officinale and Urtica dioica that reached to 74.51µg/ml to 194µg/ml [22]. After calculation, the CC_{50} for Piper betle is found to be approximately 486.404µg/ml. As mentioned above, the CC_{50} represents the extract or compound concentration required to reduce the viability of the cell by 50%. Which is why as opposed to the IC_{50}, CC_{50} will generally be better if high which directly means that the threshold for cytotoxicity will be high. When compared to previous study on Houttuynia cordata (H cordata) Thunb, CC50 of Piper betle leave extract, lower than the EA fraction of H cordata but higher than Quercetin [21]. In comparison with CC_{50} of Taraxacum officinale and Urtica dioica, the CC_{50} of Piper betle is lower [22].

The SI is the ratio between the cell cytotoxicity and the antiviral activity. Theoretically, the higher the SI the better and safer the extract or compound is against a given viral infection. SI is important because a high antiviral activity with a high cytotoxicity will lower the viability of the compound. Ideally, the antiviral activity will be high while the cell cytotoxicity remains as low as possible. Even though the CC_{50} of Piper betle lower than previous study, [21], [22] the selectivity index of Piper betle is higher due to low IC_{50}. Previous study by had shown the inhibition and cell viability of 8 natural extract at 20 µg/mL one of which, was Piper betle leave [16]. Inhibition and cell viability of Piper betle leave extract at a concentration of 20 µg/mL were 31.6% and 96.2%.16 Inhibition and cell viability results found in correspond to the results found in this study, in which the inhibition and cell viability of Piper betle leave extract at a concentration of 20 µg/mL are 40.3% and 102.1% respectively [16]. Limitations of this study were the fact that it only used one DENV serotype which was DENV-2. DENV consists of 4 serotypes thus, the next study should conduct other serotypes such as DENV-1, DENV-3 and DENV-4. Additionally, the highest concentration of extract used for MTT assay in this study does not reach 50% of cell viability. In the next study, we have to increase the concentration to more than 80 µg/mL.

5. Conclusion

Piper betle has an IC50 of 17.662 µg/ml, CC50 of 486.404µg/ml, and a SI of 24.3 which all indicates that Piper betle has a strong potential in becoming an alternative antiviral agent for dengue virus.

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