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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

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Abstract. Dengue virus (DENV) is one of the most common viral infections found Indonesia and tropical regions, and no specific antiviral for DENV. Indonesia has several of herbal medicine that were not explored of their potency as antiviral DENV. This study was done to evaluate the activity and toxicity of 4 derived fractions: Hexane (CA1), ethyl acetate (CA2), buthanol (CA3) and water (CA4) of *Cassia alata* leaf extract (CA) as an antiviral drug to DENV. The DENV was treated with various concentration of extract and added to Huh-7 it-1. The decrease of virus titer was determined by Focus assay. The toxicity of extract was measured by MTT assay. In our previous study ,we found that CA on Huh-7 cells showed IC_{50}, CC_{50} and SI values of <10 µg/mL, 323.45 µg/mL, and more than 32.3, respectively. For the fractions, CA3 showed best antiviral activity among other, with IC_{50}, CC_{50} and SI of <10 µg/mL, 645.8 µg/mL, and more than 64.5, respectively. CA and CA3 were proven to possess antiviral activity that is potent when tested against DENV-2. Future study was needed to explore the inhibition mechanism and compound of CA that have potency as antiviral drug to DENV.

1. Introduction

Dengue fever (DF) is one of the most prominent diseases in countries lying in near the equator, including Indonesia. According to the data from Ministry of Health Republic Indonesia, more than 190 000 people were diagnosed with dengue fever in the 2016 and were found to be fatal in several cases. The dengue virus had infected more than 80% of the cities in Indonesia and still continues to spread.[1] The virus has four serotypes (DENV-1, DENV-2, DENV-3, DENV-4), where the serotype DENV-2 has an approximately 75% chance of progressing the dengue fever to dengue hemorrhagic fever (DHF).[2] This serotype has the highest progression rate, where only up to 65% of the disease progresses in other dengue virus serotypes.[3]

Currently, treatment for dengue revolves around supportive intravenous fluids to improve hematocrit result. Researches have been made, aiming at the inhibition of replication and development of DENV. However, to date, there are no specific antiviral for the DENV of any serotype that can be safely used in medical centers.[3] Screening for alternative medicine in plant extracts have been done many times due to their low toxicity, low risk of side effects, and low costs. Since then, they have been successful in producing therapeutic result in a form of antibacterial, antifungal, and also antiviral.[4] One of the known plants reported for these qualities are of the Fabaceae family and Caselpiniaceae

subfamily, that is *Senna alata* or also known as *Cassia alata*. *Cassia alata* is a plant that can grow to a height of 2-3 m with the number of compound leaves. This plant is native to Indonesia and also grows in America, India, Malaysia, Brazil and Africa. Plants contain biomolecules that have varying bioactivity that can be used as a source of medicine for several different diseases. Cassia genus plants are known to contain secondary metabolites, ie antraquinon glycosides, polyphenols, flavanoids and polysaccharides.[4] Aloe-emodin, emodin, rein, crysopanol are compounds that have been isolated from *C. alata* leaves.[5] It is reported that *C. alata* has biological activity as a medicinal scab, malaria, anti oxidant, anti-bacterial wound healing against gram-positive and gram-negative bacteria, antirotavirus, and anti-fungi. [5-8]

Focusing on the leaf extracts alone, the plant contains chemical materials that is probable for developing antivirals, namely the phytosterol derivatives such as stigmasterol and β-Sitosterol, kaempferol and its derivatives, anthraquinone derivatives like aloe-emodin and chrysophanol derivatives, also rhein, ellagitinnin, phenolic acids, adenine, and xanthone.[5] The phytosterol groups for example, are known capable to inhibit absorption of cholesterol, inhibiting growth of carcinogenic cells, angiogenesis, and invasion and metastasis with an addition of trypanocidal, mosquito larvicidal as well as neutralizing agents for snake venoms. The phytosterol group also suggests have strong antioxidant abilities.[5-10] In addition, stigmasterol has a proven antiviral activity against HSC-1 while β-Sitosterol has been used as herbal medicine for its chemo preventive and neuro protective ability against BPH as well as prostate cancer through its oxidative stress mechanism.[9,11,13] Extracts mentioned above also delivers a potential antiviral property ranging from treatment against Japanese Encephalitis virus (JEV), influenza virus, enteroviruses, herpes simplex, even hepatitis virus, with some of them have also been used in antimicrobial and antifungal medicine. [9]

In this study, we screened subfraction of *Cassia alata* leaves ethanol extract and its againts DENV-2 using human cell line Huh-7 it-1 in vitro. Future study was needed to explore the inhibition mechanism and compound of CA that have potency as antiviral drug to DENV.

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2. Materials and methods

2.1. Preparation of Extract

The botanical identities of *Cassia alata* leaves extract was 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. Dried and ground leaves (1000 g) were submitted to extraction at room temperature with ethanol 70%. The solvent was removed used vaccum evaporator yielding 225.7 g of the thick extract. The extract (60 mg) was initially partitioned with n-hexane, ethyl acetate, butanol and water. The solvent was removed used vacuum evaporator yielding 4.2 g; 7.7 g; 7.1 g; 31.5 g respectively.

All of natural extracts were diluted at concentration of 100 mg/ml in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA). Centrifugation was done to remove unsolved material. The stock solution was further diluted with culture medium to the desired concentration for the assays. Stock was stored at -20^{o}C, until used. The subtitle for each extract/fraction used in this work is defined below: • CA– Crude ethanol extract Leaves • CA1 – Hexane fraction • CA2 – Ethyl acetate fraction • CA3 – Buthanol fraction • CA4 – Water fraction

2.2. Standardization of extract

Standardization of extracts and subfraction were carried out based on the total flavonoids content (TFC) of the extracts measured using aluminium chloride colorimetric assay, and also total phenolic content (TPC) measured using follin-ciocalteu colorimetric assay. For TFC determination: Extract/ subfraction solution (100 µL) and standard (quercetin) solution (50, 100, 150, 200 and 250 µL) mixed with 2 mL distilled water. After 5 minutes, the solution was then mixed with 150 µL $NaNO_2$ 5% and 150 µL $AlCl_3$ 10%. Incubate for 6 minutes, then added with 2 mL NaOH 1 N. The absorbance reading of the reaction mixture was measured at 510 nm using spectrophotometer (UV-Vis Spectrophotometer,

Japan) with methanol as blank. A standard curve was constructed using Quercetin (Sigma-Aldrich, USA). Total flavonoids content of the extracts was then compared with the standard curve, expressed in mg quercetin equivalents per 100 g dry weight (mg QE/100 g) and further calculated as % of total flavonoids content. For TPC determination: Extract/ subfraction solution (100 µL) and standard (gallic acid) solution (50, 100, 150, 200 and 250 ul) mixed with 7 mL distilled water and 0.5 mL Follin-Ciocalteau. After incubate for 8 minutes, the solution was then added with 1.5 mL Na_2CO_3 20% ,keep in room temperature for 2 hours. The absorbance reading of the reaction mixture was measured at 765 nm using spectrophotometer (UV-Vis Spectrophotometer, Japan) with methanol as blank. A standard curve was constructed using gallic acid (Sigma-Aldrich, USA). Total phenolic content of the extracts was then compared with the standard curve, expressed in mg gallic acid equivalents per 100 g dry weight (mg GA/100 g) and further calculated as % of total phenolic content.[10]

2.3. Preparation of DENV-2 and Huh 7it cells.

Dulbecco's Modified Eagle's Medium (DMEM) High glucose cat no 11965- 092 (GIBCO, UK) was used to maintain of Huh-7 it-1 cell line in this study. DMEM was added with sodium bicarbonate to maintain of the pH of the medium and 10% of Fetal Bovine Serum (FBS) (GIBCO). Cells were then incubated at 37°C with 5% CO_2 for 4-5 days until confluent. At 80-100% confluence, the cells were sub-cultured. The sub-culturing process was initiated by removing the used medium followed by rinsing the cells twice with Phosphate buffer solution (PBS) (Gibco) cat no 70011-044. Then, 1 mL of 0.25% trypsin-EDTA (GIBCO, Canada) cat no 05200-056 was added and incubated for 5 to 10 minutes at 37°C. After addition with trypsin, 1 mL of fresh medium with 10% FBS was added and mixed. The mixture was then centrifuged for 10 minutes at 1500 rpm. The supernatant was discarded while the pellet was resuspended with 2 mL of fresh medium and re-distributed into new tissue culture flask for further maintenance.

We used DENV serotype 2 strain NGC adapted in human cell line of Huh-7 it-1 cell (Dr. Y. Shimidzu, Kobe University). A monolayer of Huh-7 it-1cell in T-75 flasks were infected with DENV-2 NGC with moi of 0.5 PFU/cell and incubate at 37oC with 5% CO_2 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 1000 g for 5 minutes. Subsequently, filtered using a syringe driven 0.22 mm (Millipore, Co. Bedford MA USA). Culture supernatant was stored at -80°C and checked for the titer of dengue virus by Focus assay. [12]

2.4. Determination of cytotoxicity (CC_{50})

In vitro cytotoxicity (CC_{50}) was determined by MTT assay based on viability of Huh-7 cells after treated with extract. In 96 well flat-bottom plates (Corning, USA), we added 2×10^4 cells/well and incubated at 37°C, 5% CO_2 for 24 hours. After 24 hours, the cells were treated with various concentration of extract from 0.1 to 80 µg/mL and incubated at 37°C, 5% CO_2. 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 according to the manufacturer's instruction. 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. First, we calculate the theoretical percentage toxicity of the samples by dividing the mean blanked sample ODs by the mean blanked control ODs for each sample. The resulting percentage toxicity was provided as the "Data In" to curve fit, with the requested interpolation set to 50. The concentrations of the samples were calculated from the curve and the interpolated 50% value was provided. The absorbance was measured at 620 nm. Background absorbance of the micro plate was removed by subtraction of blank wells. We used the viability data correspond to the cytotoxic effect. The viability of the cells was determined by this following equation: The percentage viability cell, we used to determine CC_{50}. The CC_{50} was obtained from nonlinear regression analysis of concentration-effect curves by the Graph and represented the means ± standard deviation

2.5. Determination of antiviral activity (IC_{50})

A total of 2×104 cells/well were seeded into 96-wellplate and incubated at 37°C with 5% CO_2. After 24 hours, the cells were infected with DENV-2 at a moi of 1 FFU/cell which contained various concentration. After 2 hours infection, added 100 ul of DMEM + 2% FBS contained various concentration of natural extract. Plates were further incubated at 37°C for 3 days. Then we harvest and determine virus titer by focus assay (Igarashi, 1995). Briefly, a 10-fold serial dilution of supernatant was inoculated onto Huh-7 it-1 cell monolayer in duplicate wells. Absorption was carried out at 37°C in 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 35°C in 5% CO_2 for 3 days. The infected cells were stained according to previous publication (Payne AF et al, 2006) with slight modification. First, infected cells were fix with 10% formaldehide in PBS and incubate at room temperature for 1 hour. Wash the cells with PBS for 3 times. To permeabilize the cells, add 100 ul /well of 1% of P40 (P40 and incubate at room temperature for 30 minutes. Block the cell with 5% skim milk in PBS and incubate at room temperature for 1 hour. After wash, add the cell with 1/1000 of human IgG anti dengue and incubate at room temperature for 1 hour. We use 1/1000 antihuman IgG label HRP as a secondary antibody. After wash, add substrate and observed infected cell with brown color. The result from focus assay was used to determine IC50. [13]

3. Results

3.1 Standardization of Cassia alata extracts and subfraction

Table 3.1 Total flavonoid and phenolic content of *C. alata* leaves ethanol extract and subfraction

Sample	Total Flavonoid Content[1]	Total Phenolic Content[2] (2)
CA	4.87±0.46	5.39±0.03
CA1	9.43±0.08	1.96±0.02
CA2	11.22±0.3	14.79±0.04
CA3	3.54±0,92	9.65±0.06
CA4	2.01±0.12	4.46±0.03

• CA– Crude of leaves ethanol extract • CA1 – Hexane fraction • CA2 – Ethyl acetate fraction • CA3 – Buthanol fraction • CA4 – Water fraction
[1]Measured with regreesion equation y=0.0088x+0.041
[2]Measured with regression equation y= 0.1174x-0.1432

Ethyl acetate fraction showed the highest result for Total flavonoid content (TFC) 11.22±0.3 mg/g euqivalence with quercetin as a standard. And also for Total phenolic content (TPC) 14.79±0.04 mg/g equivalence with gallic acid as a standard.

3.2 Activity on inhibition of DENV by C. alata

Determination of the adequate concentration needed for the extract to produce the inhibition needed to be a potential antivirus for DENV-2 is done by introducing the *C. alata* leaves extract of six different levels of concentrations to infected hepatocarcinoma cells (Huh7 it-1), with DMSO 0.1% as a negative control. To establish a more authentic result, triplicates are made for each of the concentration and also the DMSO. After the process of focus assay and immuno staining, brownish spots will be presented when looked through the microscope. These are the brown focuses that will be counted to figure out the virus titers.

Table 3.2 Virus titer achieved after given *C. alata* extract and its subfractions

Concentration	Average of Virus Titer (FFU/mL)				
µg/mL	CA*	CA1	CA2	CA3	CA4
320	0	ND	ND	ND	ND
160	0	ND	ND	ND	ND
80	0	0	0	0.3x 10^4±0.03	0
40	0.02 x 10^4±0.01	0	0	4.2 x 10^4±0.1	0.73x 10^4±0.3
20	0.13 x 10^4±0.01	0	0	2.5x 10^4±0.3	2.03 x 10^4±0.1
10	0.20 x 10^4±0.04	0	0.2 x 10^4±0.01	5.67 x 10^4±0.1	1.5x 10^4±0.3
5	ND	0.35 x 10^4±0.1	0.2 x 10^4±0.01	8.3x 10^4±0.33	2.67 x 10^4 ±0.1
2.5	ND	1.3 x 10^4±0.33	0.83x 10^4±0.3	5.13 x 10^4± 1.5	2.93x 10^4 ±0.33
DMSO 0.1%	1.60 x 10^4±0.27	14.4 x 10^4± 1.5	7.77x 10^4± 1.5	14.4 x 10^4± 1.5	7.77 x 10^4± 1.5

*= Results of CA from our previous study

Table 3.3 Percentage of Inhibition DENV-2 after Given *C. alata* Extract and its subfractions

Concentration	Percentage of Inhibition (%)				
µg/mL	CA*	CA1	CA2	CA3	CA4
320	100	ND	ND	ND	ND
160	100	ND	ND	ND	ND
80	100	100	100	97.9	100
40	98.7	100	100	70.04	90.06
20	92.0	100	100	82.7	73.8
10	87.5	100	97.4	62.6	80.07
5	ND	96.8	97.4	42.7	65.7
2.5	ND	90.08	89.3	64.4	62.2
DMSO 0.1%	0	11.6± 0.56	0	11.6± 0.56	0

*= Results of CA from our previous study

Consequently, from the virus titer we will have gotten the percentage number of infectivity, and also percentage of inhibtion to gain the final half inhibitory concentration (IC_{50}). It is shown that the higher the concentration of extract given for the viral treatment, the percentage virus titer will also decreases. This reveals a strong viral inhibition from the extract, as it only needs a modest amount to deliver its response. Thus, explaining why extracts taken from *C. alata* is a potent inhibitor of DENV-2. IC_{50} result was 0.026 µg/mL, with logarthmic regreesion y = 6.3628 ln(x) + 73.277 and thus it will be taken to consideration that concentration needed to achieve a 50% inhibition needs to be much lower than the dose that were tested. Therefore, it will be concluded that the IC_{50} is lower than the lowest concentration tested which is <10 µg/mL. The result for hexane subfraction and ethyl acetate sub fraction The IC_{50} result is < 2.5 µg/mL. with logarthmic regreesion y = -6.665 ln(x) + 14.885 and thus it will be taken to consideration that concentration needed to achieve a 50% inhibition needs to be much lower than the dose that were tested. Study on butanol sufraction activity shown that the higher

the concentration of extract given for the viral treatment, the inhibitory effect also increases. The IC_{50} result is 6.47 µg/mL. with linear regreesion y = -2.5707 x + 14.885. Resutl for water sub fraction, shown that the higher the concentration of extract given for the viral treatment, the inhibitory effect also increases also same result with other subfraction. The IC_{50} result is < 2.5 µg/mL. with logarthmic regreesion y = -10.85 ln(x) + 51.038 and thus it will be taken to consideration that concentration needed to achieve a 50% inhibition needs to be much lower than the dose that were tested.

3.3 Toxicity of C. alata extract on the Cells

To be a potential antivirus, the extract not only has to be able to inhibit growth of the virus, but also remain harmless to the cells. Thus, half cytotoxic concentration (CC_{50}) is evaluated using MTT assay.

Table 3.4 Viability cells after treatment with *C. alata* leaves extract and its sub fractions

Concentration	Viability Cells (%)				
µg/mL	CA*	CA1	CA2	CA3	CA4
320	29.4	ND	ND	ND	ND
160	54	ND	ND	ND	ND
80	70.7	17.1	24.2	104.3	110.9
40	91.2	39.5	68.5	108.6	111.7
20	103.1	83.2	90.9	109.9	109.3
10	103.3	105.2	110.5	111.1	110.7
5	ND	112.4	111.1	108.0	112.6
2.5	ND	108.6	108.4	109.6	111.2

*= Results of CA from our previous study

Calculation use Polynomial Regression Model of the Average Cell Viability Percentage against the Extract of *C. alata*, proven that the cells become more viable in lower extract concentrations. The result of equation, $y = -0{,}0003x^2 - 0{,}2871x + 174.25$, which the CC_{50} is determined to be 323.45 µg/mL. The result for subfraction shown the same with the exatract that the cells become more viable in lower extract concentrations. The CC_{50} value of hexane, ethyl acetate, butanol and water was 47.46, 22.17, 645.8 and 311.33 µg/mL, respectively.

3.4 Selectivity Index (SI)

Selectivity index is calculated from dividing CC_{50} by IC_{50}. As a result, the final SI number is more than 32.3 for *C. alata* extract. Selectivity index for fraction can't measured clearly but in average > 18.98, >8.868, 98.29, >124.53, respectively for CA1, CA2, CA3, CA4.

4. Discussion

From this study above about standarized the extract and has been concluded that ethyl acetate extract has an high contain of total flavonoid and total phenol. From the study performed, results of IC_{50}, CC_{50}, and selectivity index has been gathered for treatment of DENV-2 by *C. alata* extract, in order to establish whether it has a potential antiviral inhibitory activity that is also non-toxic to the Huh7 cells..

Our previous study showed that the viability cells after treated with C. *alata* extract, were significant differences among each concentrations. Higer concentrations such as 320 µg/mL and 160 µg/mL showed low viability compare to control. Cells treated with 20 ug/mL or less did not show any

viability difference with control. The half-cytotoxic concentration of *C. alata extract* was 323.45 μg/mL (Prasetyo et al not publish yet). The CC_{50} value of hexane, ethyl acetate, butanol and water was 47.46, 22.17, 645.8 and 311.33 μg/mL, respectively. When we compared with the crude extract, we found that CC_{50} value of hexane and ethyl acetate were lower. In other word that hexane and ethyl acetate subfraction more toxic that crude extract. In contrary in buthanol and water subfraction we found the CC_{50} value higer that crude extract of *C. Alata*. Based on the standarized data, we found that hexane and ethyl acetat subfraction of *C alata* contain more flavonoid compound compare with crude extract, buthanol and water fraction. Report in previous report that flavonoid has cytoxicity activity.[14] The other report, showed that ethanol extracts of *C. alata* leaves exhibiting significant cytotoxic activity using the brine shrimp lethality bioassay.[15] Compounds from C alata have also exhibited anti-angiogenic activity and cytotoxic activity in breast cancer cell lines as well as protective effects against pancreatic cancer. [16,17]

The half-inhibitory concentration of *Cassia alata* was below 10 μg/mL as the lowest concentration tested yielded more than 50% inhibition. This result is quite similar with a study that was also searching for an antiviral inhibitory effects from several essential plant oils, although against yellow fever virus. Nevertheless, the trend for virus titer percentage still follows as the concentration of extract gets lower. In addition, the result shows that the extract is very potent even in the highest concentration tested, as it already produces 0% virus titer percentage. Comparing this result with the hexane and ethyl acetate subfraction, a study analyzing showed until concentration 10 ug/mL still produce 0 % virus titer percentage, and because the lowest concentration of testing was 2.5 ug/mL , we concluded the IC_{50} < 2.5 ug/mL, also either for water subfration. The result for buthanol subfraction showed the virus titer percentage increased from the highest to the lowest concentration with the IC50 < 6.47 ug/mL. Comparing the virus titer percentage wtih the capability of polyphenol-derived compounds from plants as an antiviral for DENV-2, the outcome is alike, with the virus titer percentage trend also going up as the concentration gets lower , confirming that it is potential for the extract or subfraction to be used to inhibit dengue virus growth.[18] Moreover, phenolic acids are one of the components that can be found within the *C. alata* extract and also subfraction , as explained in the above result. Though, the virus titer result is stronger correlated with the activity of antiviral extract and subfractions.

Meanwhile, the viability percentage shows that the cell toxicity became more apparent in higher concentrations as the viable cells percentage are lesser, which is true to the theory and are similar in terms of trend with other correlated researches. The number of CC_{50} is also greater in this study (323.45 μg/mL) compared with other similar studies with a CC_{50} of 252.6 μg/mL and lesser. This also might suggest that some polyphenol compounds (included in the bioflavonoids category) may differ in producing cytotoxicity effect. Having quite a high concentration to produce cytotoxicity effect means that it will be less harmful to the cell. The result for subfraction showed the buthanol and water fraction less toxic than ethyl acetate and hexane fraction. The citotoxiciy of fraction strong correlated with the major compound in the fraction. [19]

Therefore, it can be concluded that with the IC_{50} value that is <10 μg/mL and the CC_{50} value that is >320 μg/mL, it is determined that the *C. alata* extract is a suitable antiviral for DENV-2, because of its ability to produce viral inhibition even in a relatively small concentration and an agreeable cell toxicity in a concentration higher than the ones that were studied. For the sbufraction can be concluded that buthanol fraction is more suitable antiviral for DENV-2 than other fraction because IC_{50} value that is < 10 ug/mL and the CC_{50} value that is 645.8 ug/mL.

Besides that, the selectivity index received from dividing CC_{50} (323.45 μg/mL) by the IC_{50} calculated using the logarithmic regression equation (0.026 was specifically 12,440.38, which is very high. Although taking into consideration that the IC_{50} was actually less than the concentration experimented (<10 μg/mL), the SI value became > 32.3, and > 100 for buthanol extract which are still quite high as other similar studies generally have a much lower selectivity index of around the range of 1-5, suggesting that the compound in this study is most selective towards the virus and not damaging to the host.

Conclussion

Cassia alata leaves extract and buthanol subfraction are potent candidate antiviral againt DENV-2 with the IC_{50} 0.0256 and 6.47 ug/mL and CC_{50} 323.45 and 645.8 ug/mL, respectively.

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