

Drug of Action Cassia Alata Leaves Extract as Antiviral to Dengue Virus Serotype-2 *In vitro*

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ABSTRACT

Background and Objectives: Dengue viruses (DENV) is a mosquito-borne members of the Flaviridae family. To date, more than 2.5 billion people in over 100 countries are at risk of infection, and approximately 20 million infections were reported annually. Currently, There is no specific antiviral treatment available for DENV infection. Natural products possess a wide range of biological and biochemical potential. Among them, plants are one of the most important sources for discovering new drugs for therapy. Our previous study, showed that *Cassia alata* has potency as antiviral to DENV, however drug of action still unclear. **Material and Methods:** We explore the drug of action of *C. alata* leaves extract and its fraction through time of addition studies and effect of solvents with the dose based on the previous study.

Result: The most effective inhibition drug of action was determined by focus assay. Meanwhile the toxicity was measured by MTT assay. These studies demonstrated that ethanol extract of *Cassia alata* 1 mg/ml showed strong inhibition in both early step (receptor and attachment to host cells) and post infection with inhibition 96.04 % and 99.16 %. Compared with those fractions, *Cassia alata* ethanol extract has strongest inhibition DENV in every step of virus replication. **Conclusion:** *Cassia alata* ethanol extract has strongest inhibition DENV in every step of virus replication with the average of inhibition more than 95 %. Ethyl acetate and hexane has strongest inhibition with the average of inhibition 100 %.

Key words : Dengue virus, Antiviral, Cassia alata, Early step, Post infection.

INTRODUCTION

Dengue is recently reported to be the most rapidly spreading mosquito-borne viral disease and is endemic in more than 110 countries including Indonesia with two-fifths of the world population at risk. According to the data from Ministry of Health Republic Indonesia, more than 100 000 people were diagnosed with dengue fever in the 2014 and were found to be fatal in almost 1000 cases.¹ An estimated 100 million cases of dengue fever and half a million cases of dengue haemorrhagic fever (DHF) occurred annually.² The clinical presentations of DENV infections range from asymptomatic to severe illness that may lead to death if improperly managed. The symptomatic cases are categorized as undifferentiated febrile illness (UF), dengue fever (DF), dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS) and unusual dengue (UD) or expanded dengue syndrome (EDS).³

The disease is caused by dengue virus (DENV) which exists as four closely related, but antigenically distinct virus serotypes of DENV-1 to DENV-2.² This leads to many challenges in the development of a vaccine that would provide solid immunity equally to all serotypes.³ Another approach of viral diseases control is directed toward antiviral against DENV. During several decades a research has been explored natural products from medicinal plants due to the advantages of high chemical diversity of these biologically active molecules and lower cost. Many plant composition such as flavonoids, terpenoids, lignin, alkaloids,

tannins, polyphenolics, coumarins, saponins, and chlorophyllins, are reported have antiviral activity to DENV.^{4,6} Therefore, natural products from herbal medicine represent an alternative approach for treatment of DENV infections. Research for dengue antiviral has focused on the different phases of the viral lifecycle (virus attachment, viral entry, trafficking, translation or replication).

Nevertheless, only few antiviral therapies have been tested and little is known about the effects and mechanisms of the antiviral agents.⁴ Our study previously reported inhibitory activity of *C. alata* extract against DENV-2 and its toxicity to Huh-7 it-1 cells, but the inhibitory mechanism and chemical content of *C. alata* is already unknown.⁷

This study aims to evaluate drug of action *Cassia alata* leaves extract and its fraction as antiviral DENV-2 using cell culture model and knowing the compounds contained in *Cassia alata*.

MATERIAL AND METHODS

Study design

This study was an experimental study using Huh7 it-1 cell line for pursuing the DENV antiviral mechanism. Compounds identification of *C. alata* was performed using LC-MS / MS.

Preparation of extract

The botanical identities of *Cassia alata* leaves extract was determined and authenticated by the Botanical

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in Research Center for Biology LIPI. After the taxonomy identification, the *Cassia alata* leaves 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 vacuum evaporator yielding 225.7 g of the thick extract. The extract (60 g) was initially partitioned with n-hexane, ethyl acetate, butanol and water. The solvent was removed used vacuum evaporator yielding 4.2 ; 7.7 ; 7.1 and 31.5 g respectively. Extracts and fractions were diluted at concentration of 100 mg/ml in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) as a stock solution.⁷

Identification compound of *C. alata* extract using LC/MS-MS

Mass spectrometry was performed on a Xevo, G2-XS QToF (Waters MS Technologies). Ionisation type is ESI. The scan range was from 100 to 1200 m/z. The capillary and cone voltage was set at 0.8 kV and 30 kV, respectively and was used positive electro spray mode. The desolvation gas was set to 1000 L/h at a temperature of 500°C and the cone gas was set to 50 L/h and the source temperature was set to 120°C. The UPLC analysis was performed using a Waters Acquity Ultra Performance LC system. Chromatographic separation was carried out on an ACQUITY UPLC HSS T3 C18 column (100 mm x 2.1 mm, 1.7 µm) at a column temperature of 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile), with gradient polarity from 95:0.5 (A: B) to 0.5:95 (A: B). The flow rate was set at 0.3 mL/min. The column and auto sampler were maintained at 40 °C and 20 °C, respectively. The injection volume was 1 µL. The data acquisition and processing were performed using UNIFI. The parameter used was retention time (RT) in the range of 1- 15 min. Determination of the dominant compound was carried out with semi quantitative determination based on the high intensity of the compound on the LC-MS/MS graph.

Preparation of DENV-2 and Huh-7-it-1

We used DENV serotype 2 strain New Guinea C (DENV-2 NGC) adapted in Huh-7-it-1 cell line. A monolayer of Huh-7-it-1 cell in T-75 flasks were infected with DENV-2 NGC with moi of 0.5 PFU/cell and incubate at 37°C with 5% CO₂ 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. Culture supernatant was stored at -80°C and checked for the titer of dengue virus by Focus assay.^{8,9}

Determination of cytotoxicity

In vitro cytotoxicity was determined by MTT assay based on viability of Huh-7-it-1 after treated with extract. In 48 well flat-bottom plates (Corning, USA), we added 5 × 10⁴ cells/well and incubated at 37°C, 5% CO₂ 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% 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 according to the manufacturer's instruction. The absorbance reading of each well was measured using micro plate reader at 490 nm.⁷ 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. We used the viability data correspond to the cytotoxic effect.^{8,10}

Determination mechanism of antiviral action of CA and its fractions

To determine mechanism of antiviral action that would explain the

possible inhibitory step(s) in the viral life cycle, we used published method with slight modification.^{11,12} We carried out a time-of-addition study with the concentration of CA and its fractions of 10 mg/mL and monolayer (5×10⁴ cells/well) Huh-7-it-1 at 48 well plate.

For inhibition of DENV receptor (i), we added extract or fractions into cell for 2 hours before DENV infection at 37°C with 5% CO₂. The residual extract or fraction were removed and washed. Then DENV-2 was infected into cells for 2 hours at 37°C with 5% CO₂. After virus adsorption, the remain virus was removed and the medium without extract was added and incubated for 48 hours. For entry step experiment (ii), DENV-2 was mixed with extract or fractions at concentration of 10 µg/mL, then infected to the cell for 2 hours at 37°C with 5% CO₂. After virus adsorption, the residual virus and test sample were removed and washed. Then the cells were added with medium and incubated at 37°C with 5% CO₂ for 48 hours. Post entry step experiment (iii), DENV-2 was infected to the cells for 2 hours, and removed the residual virus. The cells were added with medium containing of Extract and its fraction and incubated for 48 hours. The whole step experiment or pre-post experiment (iv) was combining of pre and post experiment. The DENV-2 were treated with extract or its fraction prior infection and followed by addition of medium with medium containing of extract and its fraction As a positive control: we infected cells with DENV-2 and the cells were added with medium without extract of its fraction for 48 hr.

After 48 hours, then we harvest and determine virus titer by focus assay with the same method above.^{8,11} The result from focus assay was used to determine percentage of virus inhibition.^{8,13}

RESULTS AND DISCUSSIONS

Profile of LC-MS/MS of *Cassia alata* leaf extract and fractions

Chemical constituents of *Cassia alata* leaves were reported: aloe emodin, emodin, ω-hydroxyemodin, lunatin, physcion, ziganin, apigenin, 7,4'-dihydroxy-5- methoxyflavone, diosmetin, kaempferol, luteolin, trans-dihydrokaempferol, trans-resveratrol.^{14,15} *C. alata* leaf extract has been reported to have various pharmacological activities including antibacterial, cytotoxicity, anti-inflammatory, antidiabetic, antihepatotoxic and hepatoprotective effects, antiseptic, antiviral and exhibited strong DPPH radical scavenging activities.¹⁶⁻¹⁹

Whereas from the measurement data using LC-MS/MS (Figures 1-5), it is known that *Cassia alata* leaves contain 5,7,2',5'- Tetrahydroxyflavone, Daturametelin H, Kaempferol-3,7- diglucoside, β-sitosterol-3-0-β-D-glucopiranoside, Digitopurpon, 25-Dehydroxy-24-acetate alisol, 3,3',5,5-Tetramethoxy-trans-stilbene, Deoxycholic acid. It can be seen that several differences compounds measured by LC-MS/MS with previous research reports suggest that a number of things that affect the difference in compound content are the location where the plants grow, the time of harvesting leaves and solvents and the extraction process.²⁰ There are differences in the dominant compounds in each fraction due to differences in the polarity of each fraction. Polar compounds will be dominant in the more polar fraction and conversely the non-polar compound will be dominant in the more non-polar fraction (Table 1, Figures 1-5).

Cytotoxicity of *C. alata* extract and fractions on the Huh7-it-1 cell line

Based on previous tests it was known that CA, CA1, CA2, CA3, CA4 had DENV antiviral activity on Huh 7-it-1 cell with the focus assay method, where there was a decrease in the focus on dengue-infected Huh 7-it-1 cells.⁷ Moreover, we want to investigate antiviral DENV mechanisms from extracts of CA, CA1, CA2, CA3, and CA4 through time addition

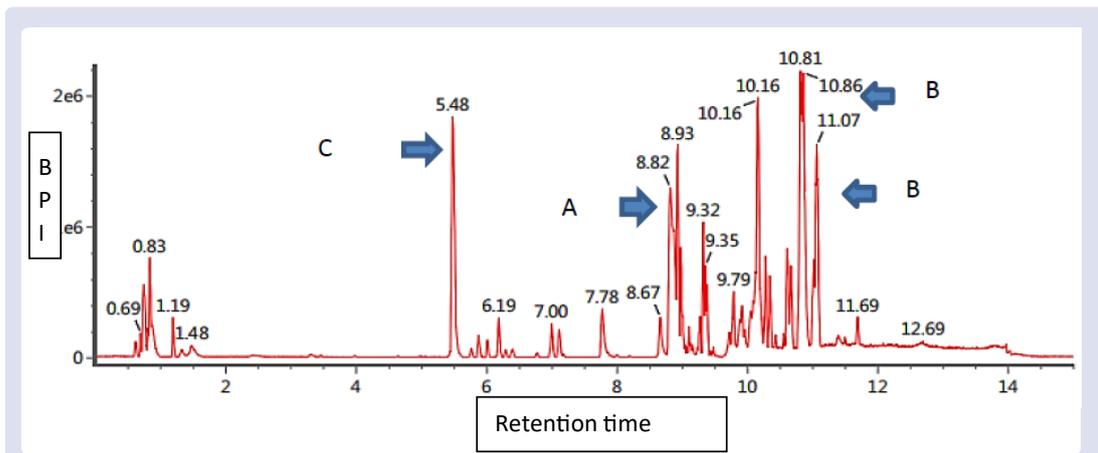


Figure 1: LC-MS of *C. alata* ethanol extract A. 5,7,2',5'- Tetrahydrox-flavone, B. Daturametelin H, C. Kaempferol- 3,7- diglukoside.

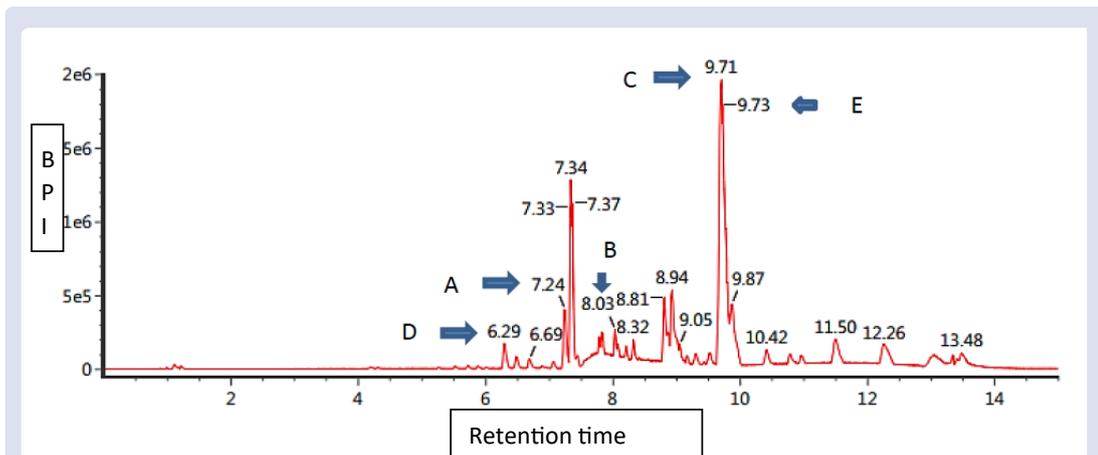


Figure 2: LC-MS *C. alata* n-heksane extract A. 5,7,2',5'- Tetrahydrox-flavone, B. Clinopodiside F, C. Daturametelin H, D. Kaempferol- 3,7- diglukoside, E. α -sitosterol-3-0- δ -D-glukopiranoside.

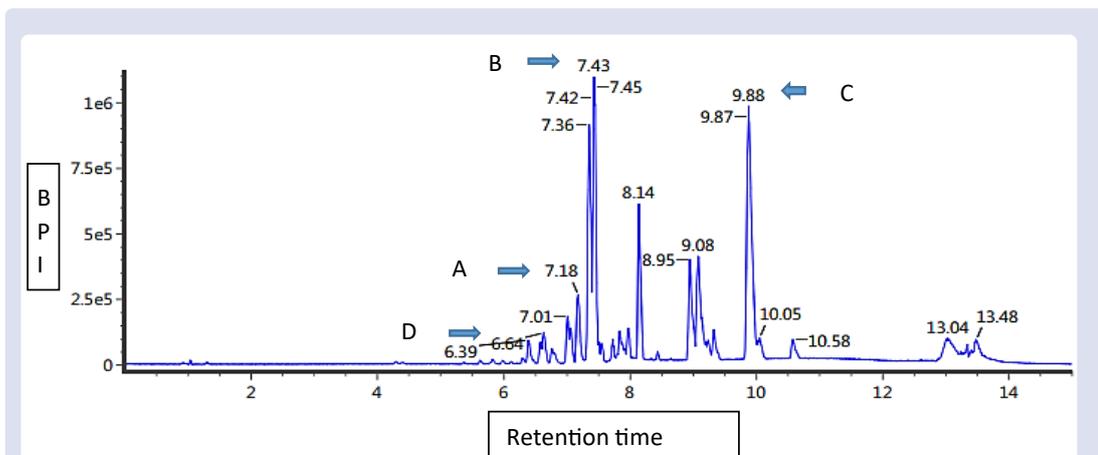


Figure 3: LC-MS *C. alata* etil asetat fraction A. 5,7,2',5'- Tetrahydrox-flavone, B. Digitopurpon, C. Daturametelin H, D. Kaempferol- 3,7- diglukoside.

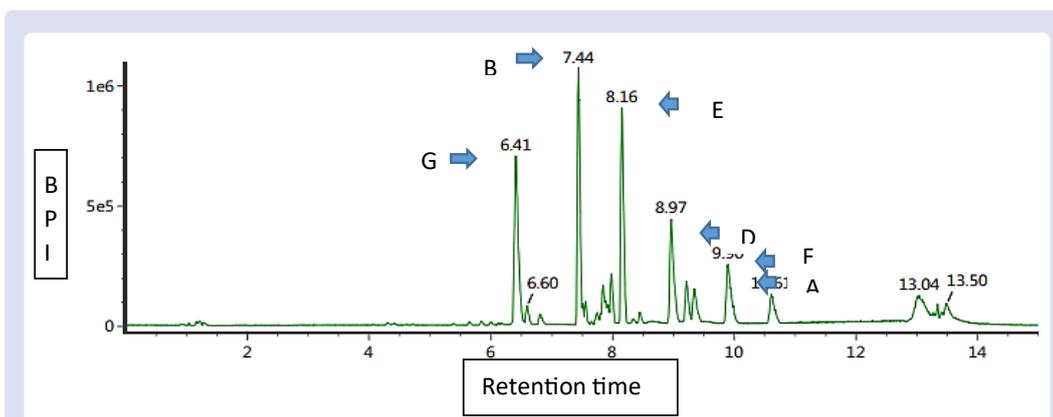


Figure 4: LC-MS *C. alata* butanol fraction A. 25-Dehydroxy-24-acetate alisol B. 3,3',5,5'-Tetramethoxy-trans-stilbene, C. 5,7,2',5'- Tetrahydrox-flavone, D. Bis(2-ethylhexyl)phthalate, E. Deoxycholic acid, F. Epianhydrobelachinal, G. Kaempferol- 3,7- diglukosida.

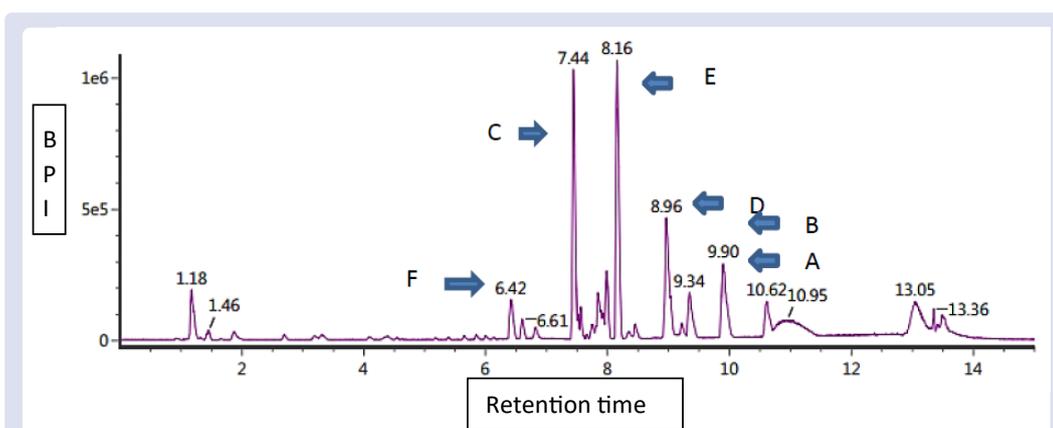


Figure 5: LC-MS *C. alata* water fraction A. 21-O-Methyltoosendanopentanol, B. 25-Dehydroxy-24-acetate alisol, C. 3,3',5,5'-Tetramethoxy-trans-stilbene, D. Bis(2-ethylhexyl)phthalate, E. Deoxycholic acid, F. Kaempferol- 3,7- diglukosida.

studies. To be a potential antiviral drug the extract not only has to be able to inhibit growth of the virus, but also remain harmless to the cells. Therefore, we determine the toxicity after the cells were treated with extract and its fraction at concentration of 10 µg/mL using MTT assay. CA, CA1, CA2, CA3 and CA4 showed no toxic effect to the cell based on the percentage of viability cell of Huh7 it-1 (Table 2).

Time of addition studies *cassia alata* leaves extract and fractions

Dengue virus (DENV) infection begins with the attachment of a viral capsid protein to the receptor (attachment) which in turn mediates endocytosis through the formation of endosomes with low pH and induces fusion of DENV.^{21,22} This stage can be a target of antivirals where obstacles to the viral envelope or parts of cell receptors will prevent infection. In this study the resistance to virus particles was directly tested by exposing CA, CA1, CA2, CA3, CA4 to DENV-2 isolates, then inoculating the cells. The result of viral titers at the addition of CA at a dose of 10 µg/ml, inhibition reached 99.28%. While the virus inhibition after the addition of CA1, CA2, CA3, CA4 were 92, 63, 85 and 89%, respectively. CA has the most potent activity in damaging the integrity of membranes on the surface of virus particles.

The next test is the addition of CA, CA1, CA2, CA3, CA4 extract to the medium after the cell is infected. The test results provide inhibition of 99.06% at the concentration of CA 10 µg/ml. Whereas for fractions of CA1, CA2, CA3 and CA4 fractions give inhibitions of 100, 100, 88, 87% respectively (Figure 6).

The non-polar fraction of hexane (CA3) and ethyl acetate (CA2) gave better results in the post-infection mechanism. It was estimated that the content of non-polar compounds found in the fraction of CA2 were an active compounds to inhibit DENV. Analysis with LC-MS / MS, known that the CA2 and CA3 contain several same compounds i.e. ,7,2',5'- Tetrahydrox-flavone, Daturametelin H and Kaempferol-3,7-diglukoside. In this mechanism, after infection, antiviral is continuously present in the culture medium. In a previous study reported tests on vero cells added with medium and curcumin and incubated for one day showed that curcumin can accumulate in cells when detected using a confocal microscope.²³

This result indicates CA, CA1, CA2, CA3, CA4 can inhibit DENV replication in cells. CA is an ethanol extract that is semipolar in nature, presumably the nonpolar part of CA which is lipophilic can penetrate the cell membrane.²² Replication of the viral genome occurs

Table 1: Identification compound of ethanol extract of *C. alata*, n-hexane fraction, ethyl acetate fraction, butanol fraction and water fraction.

Extract/Fraction	Compounds	Retention time (Rt)	Molecule weight	Intensity
Ethanol Extract	5,7,2',5'- Tetrahydrox-flavone	8,82	287,05	++
	Daturametelin H	10,86	621,31	+++
	Kaempferol- 3,7- diglukosida	5,48	611,16	++++
n-Heksana Fraction	5,7,2',5'- Tetrahydrox-flavone	7,24	287,06	+++
	Klinopodisida F	8,03	991,55	+
	Daturametelin H	8,94	621,3	+
	Kaempferol-3,7-diglukosida	6,29	633,14	+
Ethyl acetate fraction	□-sitosterol-3-0- -D-glukopiranosida	9,73	559,43	++++
	5,7,2',5'- Tetrahydrox-flavone	7,36	287,06	++++
	Digitopurpon	7,43	271,06	++++
	Daturametelin H	9,88	621,3	++++
	Kaempferol-3,7-diglukosida	6,64	633,14	+
	25-Dehydroxy-24-acetate alisol A	10,61	537,36	
Butanol fraction	3,3',5,5'-Tetramethoxy-trans-stilbene	7,44	301,14	+
	5,7,2',5'-Tetrahydroxy-flavone		287,06	++++
	Bis(2-ethylhexyl)phthalate		413,27	++
	Deoxycholic acid	8,16	393,3	++++
	Epianhydrobelachinal	9,9	469,33	+
	Kaempferol-3,7-diglucoside	6,41	611,16	+++
	21-O-Methyltoosenddanopentanol	10,62	521,38	+
Water fraction	25-Dehydroxy-24-acetate alisol A	9,9	637,36	+
	3,3',5,5'-Tetramethoxy-trans-stilbene	7,44	301,14	++++
	Bis(2-ethylhexyl)phthalate	8,96	413,27	++
	Deoxycholic acid	8,16	393,3	++++
	Kaempferol-3,7-diglucoside	6,42	633,14	+

Table 2: Viability cells after treatment with *C. alata* leaves extract and its sub fractions.

Treatments	Viability Cells (%)				
	CA	CA1	CA2	CA3	CA4
Reseptor	68,91 ± 15,23	75,37 ± 6,49	109,64 ± 12,12	110,32 ± 4,59	143,79 ± 4,64
Whole	75,54 ± 11,76	97,21 ± 0,77	113,39 ± 7,6	171,35 ± 6,07	98,34 ± 18,87
Post	121,27 ± 45,07	100,58 ± 00,4	144,19 ± 22,13	262,28 ± 4,56	139,67 ± 22,13
Pre	118,08 ± 10,93	95,33 ± 5,17	126,71 ± 12,72	149,59 ± 25,03	131,08 ± 0,37

CA- Crude ethanol extract • CA1 - Hexane fraction • CA2 - Ethyl acetate fraction • CA3 - Buthanol fraction • CA4 - Water fraction.

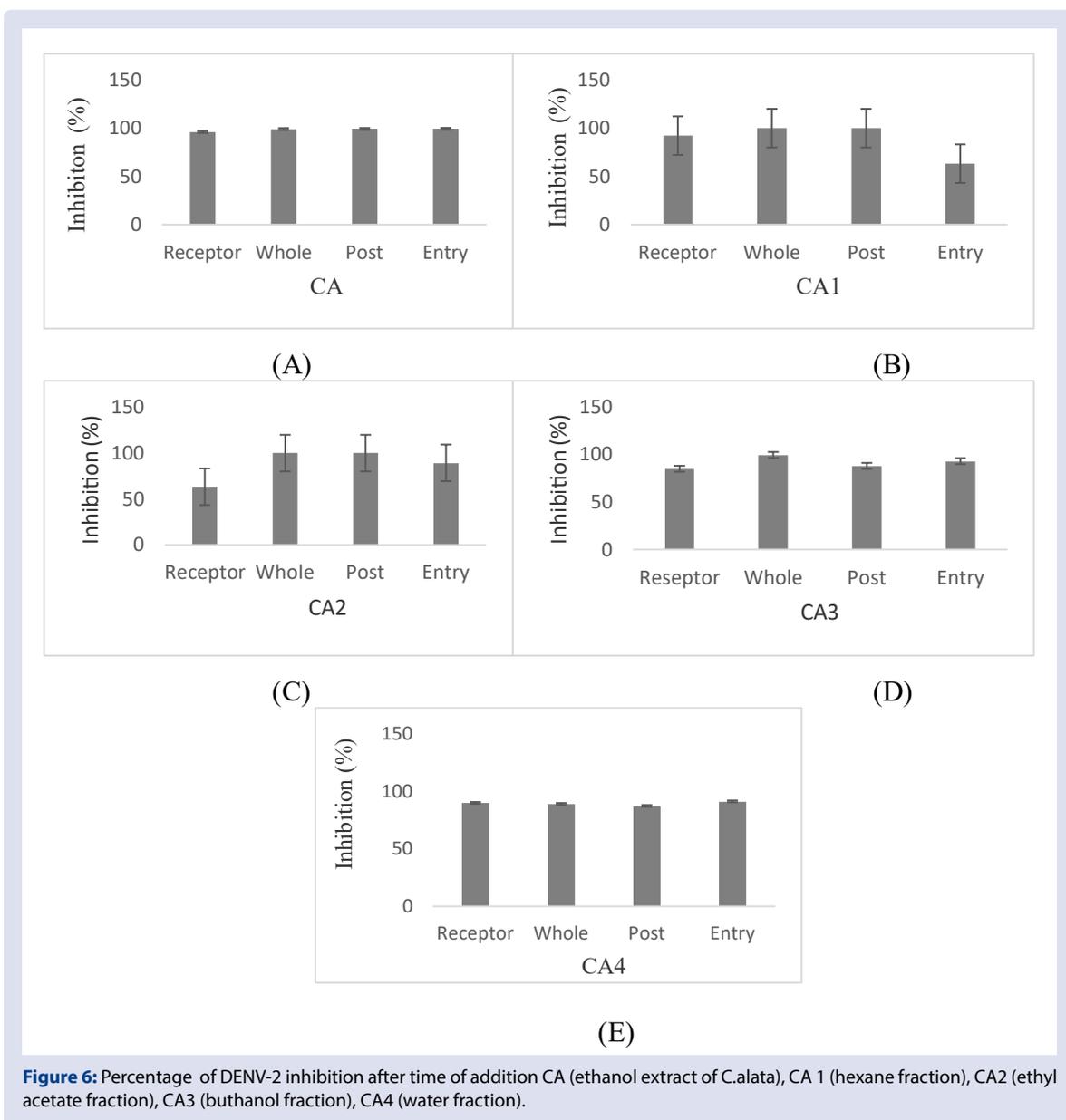
in the cytoplasm, then towards the endoplasmic reticulum where viral particles are assembled, antiviral accumulation in the cell cytoplasm is thought to interfere with the DENV replication process.²⁴

In the treatment where exposure of CA, CA1, CA2, CA3, CA4 before infection to DENV-2 and the addition of CA to the medium after the infected cell gave a maximum barrier of 99.06%, whereas when compared to the results of inhibition at the attachment and post-infection stages CA was concluded to be better inhibited at the post-infection stage (Figure 6).

While in the treatment of CA exposure to cells or receptor blocks, the inhibition percentage was 96.04% (Figure 6). The possible inhibition

mechanism of CA in this way is on the cell membrane by the accumulation of CA, as well as things related to replication in the cell. These results indicate that to be able to inhibit DENV replication in cells exposed to CA for 2 hours, CA requires a concentration of 10 µg/ml.

These studies demonstrated that ethanol extract of *Cassia alata* showed strong inhibition in both early step (receptor and attachment to host cells) and post infection with inhibition 96.04 % and 99.16 % (Figure 6). Compared with those fractions, *Cassia alata* ethanol extract has strongest inhibition DENV in every step of virus replication. We supposed the antiviral activity of CA extract is influenced by the



compounds contained in the extract. Kaempferol -3,7-diglucoside is the highest intensity compound contained in CA extracts compared to fractions. From the previous research, it was reported that kaempferol glycosides are good candidates as antiviral for 3a channel proteins of coronaviruses.²⁵ To know the mechanism more deeply especially which protein of DENV was inhibited by CA and fraction could be investigated through bioinformatic and enzymatic testing of each protein DENV.

CONCLUSION

Cassia alata ethanol extract has the strongest inhibition of DENV in every step of virus replication with an average inhibition of more than 95%. Ethyl acetate and hexane have the strongest inhibition with an average inhibition of 100%.

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CONFLICTS OF INTEREST

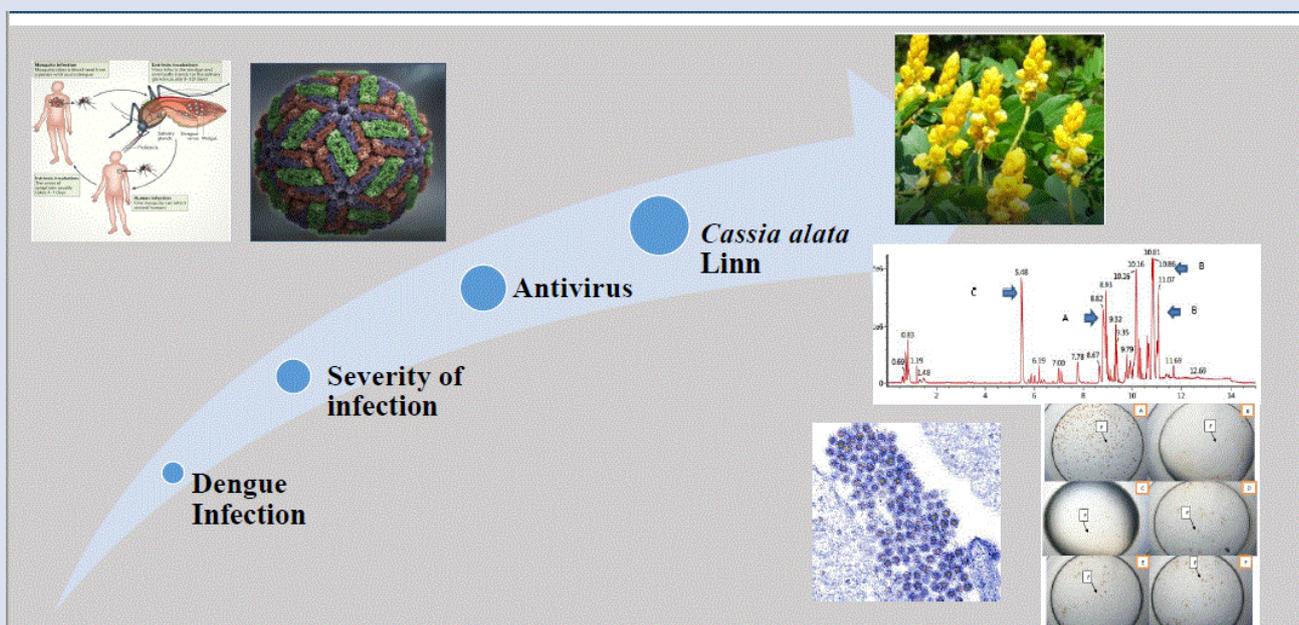
The authors declare no conflicts of interest.

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GRAPHICAL ABSTRACT



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