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# *Cassia alata* butanol fraction anti-inflammatory effect on early expression of NFκB and cytokines in DENV-1 infected PBMCs

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**ABSTRACT**

Cytokine storms impact endothelial cells in vascular tissue, leading to plasma leakage during dengue infection. Several cytokines play important roles in dengue virus (DENV) infection such as TNF-α, IL-6, and IL-10. TNF-α and IL-6 were produced from the NFκB pathway. Prior investigation suggests the potential antiviral activity of butanol fraction derived from *Cassia alata*, but its potential as anti-inflammation remains unexplored. In this study, peripheral blood mononuclear cells from a healthy donor were infected with DENV-1 and treated with *C. alata* at 2 × $IC_{50.}$ mRNAs expression was measured by real-time PCR at two time points: 2 and 24 hours post-treatment. The results showed that the expression of NFκB remained relatively constant between 2 and 24 hours post-treatment, while TNF-α and IL-6 in the DENV-1-infected-group were elevated at 24 hours post-treatment. In contrast, IL-10 decreased at 24 hours post-treatment. At 2 hours after treatment, expression of NFκB, TNF-α, IL-6, and IL-10 decreased compared to the untreated group, by 0.084, 0.528, 3.519, and 0.445 fold, respectively. At 24 hours, NFκB expression was slightly down-regulated, while TNF-α, IL-6, and IL-10 were slightly up-regulated. The mRNA expression changes were not statistically significant, but the results showed that the effect of *C. alata* leaves butanol fraction was stronger at 2 hours post-treatment in the downregulation of TNF-α, IL-6, and IL-10 expression. Further study is needed to evaluate the potential and mechanism of *C. alata* as an anti-inflammation.

## INTRODUCTION

Severe dengue is a potentially fatal complication, due to plasma leakage, fluid accumulation, respiratory problems, bleeding, and organ damage, thereby increasing the risk of death [1]. In dengue virus (DENV) infection, the early immune responses play a dual role, involving innate immunity and an inflammatory response. Both collaborate to mediate the body's protection, while at the same time, they can worsen the severity of the infection, namely when excessive cytokine production occurs and triggers a cytokine storm [2]. Cytokine storms directly affect endothelial cells in vascular tissue by increasing capillary permeability resulting in plasma leakage [3]. Therefore, the most effective antiviral treatment not only involves agents that can inhibit virion formation, but also can inhibit the development of cytokine storm [4].

In DENV infection, activation of the NFκB pathway also occurs when pattern recognition receptors recognize pathogen-associated molecular patterns. This activation induces the transcription of proinflammatory cytokines, chemokines, and additional inflammatory mediators in various immune cell types. There are various pro-inflammatory cytokines produced from the NFκB pathway, namely IL-1, IL-2, IL-6, IL-8, IL-12, and TNF-α [5]. Some of them are cytokines that have an essential role in the severity of DENV infection, such as TNF-α, IL-8, IL-6, and IFN-γ, which can disrupt of adhesion molecules [6–8], adherence junction [9], and tight junctions [10]. This disturbance results in increased

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endothelial permeability [11], allowing plasma to reach the intercellular cell junctions below, resulting in leakage and plasma being able to escape from the blood vessels [12,13]. IL-10 is an anti-inflammatory cytokine that is a marker for DENV infection [14]. IL-10 exerts anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines by the Janus kinase 1/ Signal transducer and activator of transcription 3 (JAK1/STAT3 pathway) [15].

*Cassia alata* is an herbaceous plant from the *Leguminoceae* family that is widespread and cultivated for medicinal purposes such as diabetes, diarrhea, microbial infections, malaria, cure fever, healing dermal wounds, and so on. Several scientific studies have also been carried out regarding the therapeutic activity of *C. alata* as an anti-fungal [16,17], anti-bacterial [18], antioxidant, antiviral [19], anti-diabetic [20], anti-inflammatory, anti-lipogenic, anti-hyperlipidemic, anthelmintic, and anti-malaria [21]. These potentials are supported by the content of bioactive chemical compounds such as phenol (rhein, chrysophanol, kaempferol, aloeemodin, and glycosides), anthraquinones (alatinone and alatonal), fatty acids (oleic, palmitic, and linoleic acids), steroids, and terpenoids (sitosterol, stigmasterol, and campesterol) [21,22].

Angelina *et al*. [23] show that the ethanol fraction of *C. alata* leaves originating from Jakarta has the most robust DENV inhibitory ability at each stage of virus replication with an average inhibition >95% [23]. *In vitro* studies on Huh-7it-1 cells infected with DENV-2 showed that the butanol fraction of *C. alata* leaves originating from Jakarta (containing active compounds in the form of kaempferol-3,7-diglucoside, tetrahydroxy-flavone, and deoxycholic acid) had activity potent antiviral with an $IC_{50}$ value of 6.47 g/ml and SI 98.29 g/ml), making it a potential candidate for DENV antiviral [24]. This study investigated the anti-inflammatory properties of the butanol fraction derived from the ethanol extract of *C. alata* leaves, cultivated in Solo, Java Island, Indonesia, in the context of DENV-1 infection. The NFκB pathway was examined, which is crucial for producing several pro-inflammatory cytokines such as IL-6 and TNF-α that lead to plasma leakage. The expression of IL-10, an anti-inflammatory cytokine, was also considered. Therefore, this research focused on analyzing NFκB, TNF-α, IL-6, and IL-10 expression levels.

## MATERIAL AND METHODS

### Materials

#### *Chemicals and extract*

The butanol fraction of *C. alata* leaves ethanol extract used in this study was obtained from PT. Konimex, Solo, Indonesia, dissolved in dimethyl sulfoxide (DMSO; Thermo Scientific, USA). The stock with a final 100 mg/ml concentration was stored at 4ºC until use. In the inhibitory concentration 50% ($IC_{50}$) and cytotoxicity concentration 50% ($CC_{50}$) tests, the extract was diluted with 2% FBS MEM (Gibco, USA); and in the anti-inflammatory activity test, the extract was diluted with 2% FBS RPMI 1640 (Gibco, USA) until the desired concentration was obtained.

#### *Cells and viruses*

The cells used for DENV propagation, $IC_{50}$ and $CC_{50}$ determination were Vero E6 C1008 cells from African green monkey kidneys cultured in MEM medium (Gibco, USA) supplemented with Fetal Bovine Serum (Sigma, USA) and Antibiotic Antimycotic Solution (10,000 units penicillin, 10 mg streptomycin and 25 µg Amphotericin B per ml) (Sigma-Aldrich, USA). The cells used for anti-inflammatory test were peripheral blood mononuclear cells (PBMC) from a donor who was not being infected with DENV, cultured in RPMI 1,640 media (Gibco, USA) (ethical clearance from Health Research Ethics Committee, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia number KET-1449/UN2.F1/ETIK/PPM.00.02/2023). The virus used was DENV serotype-1 (DENV-1) strain IDS 11/2010 (collection of Department of Microbiology, Faculty of Medicine, Universitas Indonesia), propagated in Vero E6 C1008 cells.

### Methods

#### *The $IC_{50}$, $CC_{50}$, and selectivity index determination*

To determine $IC_{50,}$ a dose-dependent test was carried out. It is necessary to determine the dose to be used in the anti-inflammatory test study and follow published procedures [24] with slight modifications. Vero cells were seeded in 96-well plates with a concentration of $2 \times 10^4$ cells in 100 µl/well then incubated for 24 hours at 37ºC, 5% $CO_2$ until they reached 80% confluence. Virus at multiplicity of infection (MOI) 0.5 FFU/cell were mixed with *C. alata* extract butanol fraction with various concentrations (2, 1, 0.25, and 0.125 µg/ml) in triplicate. DMSO at 0.1% was used as a negative control.

To determine the DENV-1 titer, a focus assay was carried out using Vero E6 C1008 cells in 96-well plates [24,25]. After incubation for 2 days, the cells were stained and foci were counted under an inverted microscope [25].

Determination of the $CC_{50}$ value was carried out using the MTT ((3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida) test based on references from previous research [23,26], in two independent experiments. $CC_{50}$ is commonly used in toxicology studies as an indicator of a compound's cytotoxicity to a cell population [27]. The initial experiment utilized 320, 160, 80, and 40 µg/ml dose ranges. The subsequent experiment employed 240, 120, 60, and 30 µg/ml doses. The absorbance readings were carried out at a wavelength of 490 nm with blank wells without cells (added with 100% DMSO) as background. The cell viability of the treatment group was compared with the control group in percentage form using the formula:

$$Cell\ viability\ percentage: \frac{\text{Absorbance 490 nm} - \text{background}}{\text{The average control absorbance}} \times 100\%$$

From the $IC_{50}$ and $CC_{50}$ values, the selectivity index (SI) value can be determined which is the quotient of $CC_{50}/IC_{50}$ [28].

### Anti-inflammation assay

*PBMC Isolation*

Twelve ml of blood was taken in EDTA tubes from a donor who met the inclusion criteria (no fever for 3 weeks,

NS1 negative, and IgM anti-DENV negative). The PBMC isolation procedure was based on references [29,30] with slight modifications by using the cell scrapper to detach the adherent PBMC from the bottom of the flask. PBMCs were incubated in a T75 flask at 37°C with 5% $CO_2$. After 2 hours of incubation, non-adherent PBMC (those not sticking to the flask) were discarded. Then, 4 ml of RPMI with 10% FBS was added to the flask, and using a cell scrapper the adherent PBMC was collected and transfered to a 15 ml tube. The adherent PBMC was counted using an improved Neubauer hemocytometer The PBMC was then planted in a 96-well flat bottom microplate at a concentration of $2 \times 10^5$ cells/well and incubated at 37°C with 5% $CO_2$. After 24 hours, the medium was removed and treated according to the treatment groups.

*Anti-inflammatory test*

The anti-inflammatory experiment was done on PBMC infected by DENV-1 based on the ADE model (Widodo *et al*., manuscript under review for publication). PBMC cells were infected with DENV-1 final MOI 0.5 FFU/cell, 50 µl/well, then incubated for 2 hours at 37ºC with 5% $CO_2$ and agitation every 30 minutes. After that, *C. alata* extract treatment with a final concentration of $2 \times IC_{50}$ in 50 µl/well was added, followed by 2 hours and 24 hours incubation. The RNA from the cells was then extracted to determine cytokine mRNA expression. Concanavalin A 1 µg/ml was used as a positive control (data not shown). PBMC without DENV infection was used as a negative control.

*RNA extraction*

RNA extraction was performed using the Total RNA Mini Kit (Blood/Cultured Cell) (Geneaid, Taiwan), following the manufacturer's instructions. RNA was eluted in 40 µl RNase-free water. RNA concentration was measured by nanodrop at wavelengths of 230 nm, 260 nm, and 280 nm. The purity index was measured as 260/280 and 260/230. Next, cDNA synthesis was immediately carried out, the remaining RNA was stored at −80ºC.

*cDNA synthesis*

cDNA was synthesized with the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The amount of RNA template used for cDNA synthesis was 40 ng. A total volume of cDNA synthesis mix (20 µl) consisted of 40 ng RNA template, 1 µl random hexamer primer (Thermo Scientific, USA), nuclease-free water (NFW), 4 µl 5X reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl 10 mM dNTP Mix, and 1 µl RevertAid M-MuLV RT. cDNA synthesis program followed the manufacturer's instructions. The cDNA product was then stored at −20ºC and used as real time PCR template.

*Real-time PCR assay*

Real-time PCR was carried out to analyze target cytokine mRNA using the PowerUp SYBR Green Master Mix kit (Applied Biosystem, USA). Cytokine mRNA (TNF-α, IL-6, IL-10, and NFκB) was quantified by real-time PCR using specific primers and previously synthesized cDNA templates.

**Table 1.** The primer sequences of inflammation-related genes utilized in real-time PCR.

| Genes | Primer sequences | References |
|---|---|---|
| NFκB | F: ATCCCATCTTTGACAATCGTGC | [31] |
| | R: CTGGTCCCGTGAAATACACCTC | |
| TNF-α | F: TGCTTGTTCCTCAGCCTCTT | [32] |
| | R: ATGGGCTACAGGCTTGTCACT | |
| IL-6 | F: GTACATCCTCGACGGCATC | [32] |
| | R: AGCCACTGGTTCTGTGCCT | |
| IL-10 | F: GCCTAACATGCTTCGAGATC | [33] |
| | R: TGATGTCTGGGTCTTGGTTC | |
| β-actin | F: AGAAAATCTGGCACCACACC | [32] |
| | R: CTCCTTAATGTCACGCACGA | |

A total of 1 µl of cDNA was added to 10 µl of master mix, with 1.25 µl of forward primer and 1.25 µl of reverse primer for each target gene (Table 1), as well as 6.5 µl of NFW. Real-Time PCR conditions included: (i) pre-incubation at 95°C for 5 minutes; (ii) 40 amplification cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds (same annealing temperature for all primers), and extension at 72°C for 20 seconds; and (iii) melting curve and cooling step.

Relative mRNA expression was analyzed by the delta delta CT method (ΔΔCT) so that the fold changes value is obtained. The fold change value is obtained from several stages of analysis starting from calculating ΔCT. The ΔCT value is the difference in CT value between the target gene and the reference gene (in this study the reference gene used was β-actin). Then continued by calculating ΔΔCT. ΔΔCT is the difference in ΔCT between the target sample (treatment sample) and the reference sample (negative control) [34]. The final result obtained is the fold changes of target gene expression in the target sample relative to the reference sample, normalized to the reference gene. The relative gene expression is made to 1 for the reference sample because ΔΔCT is equal to 0 and, therefore, $2^0$ is equal to 1 [35]. From the results of ΔΔCT obtained, the value of $2^{-\Delta\Delta CT}$ is then determined, to determine the fold changes of target gene expression in the sample.

**Data analysis**

Statistical analysis was carried out using GraphPad Prism 9 software. Statistical differences between groups were analyzed using *T*-Test and one-way analysis of variance to determine the significance of mean differences between the treatment group and the control group.

## RESULTS

**$IC_{50}$, $CC_{50}$, and selectivity index of *C. alata* butanol fraction**

The $IC_{50}$ value obtained was 5.46 µg/ml. This concentration was used as the extract dose in the anti-inflammatory test (we used $2 \times IC_{50}$ concentration). From 2 independent experiments, we found that the average of $CC_{50}$ value was 101.96 µg/ml. The SI was determined by calculating the ratio between $CC_{50}$ and $IC_{50}$. The SI value for the butanol

fraction of *C. alata* leaf extract in Vero E6 C1008 cells in this study was 18.67.

**Anti-inflammatory activity of *C. alata* butanol fraction in PBMC cells**

The anti-inflammatory activity of the butanol fraction of *C. alata* was analyzed using delta delta CT (ΔΔCT) to obtain the fold changes value of the target cytokine expression.

At 2 hours of treatment, it was observed that there was a downregulation in the expression of NFκB) and all cytokines (TNF-α, IL-6, and IL-10) in the group treated with a butanol fraction of *C. alata* leaf extract 2 × $IC_{50}$. The downregulation in expression of the transcription factor NFκB was slight (0.084-fold change). The cytokines TNF-α, IL-6, and IL-10 were down-regulated by 0.528; 3.519; and 0.445-fold change, respectively (Fig. 1). However, these downregulations were not statistically significant. The most substantial downregulation observed is in IL-6, suggesting that the fraction of *C. alata* leaf extract 2 × $IC_{50}$ exerts a particularly strong influence on IL-6 levels within the first 2 hours of treatment. This indicates that the butanol extract may be highly effective in rapidly reducing IL-6 expression during this time frame.

At 24 hours of treatment, it was observed that there was a slight downregulation on the expression of NFκB in the group treated with a butanol fraction of *C. alata* leaf extract 2 × $IC_{50}$, while TNF-α, IL-6, and IL-10 were slightly up-regulated

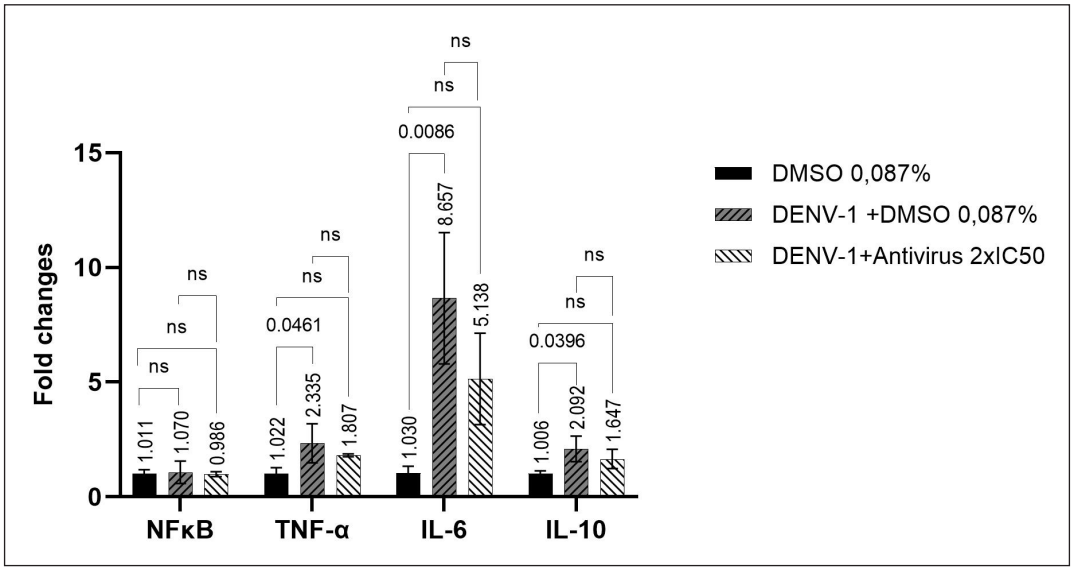


**Figure 1.** The expression of NFκB and cytokines in 2 hours post-treatment butanol fraction of *C. alata* leaf extract 2 × $IC_{50.}$ Relative mRNA expression was analyzed by the delta delta CT method (ΔΔCT).

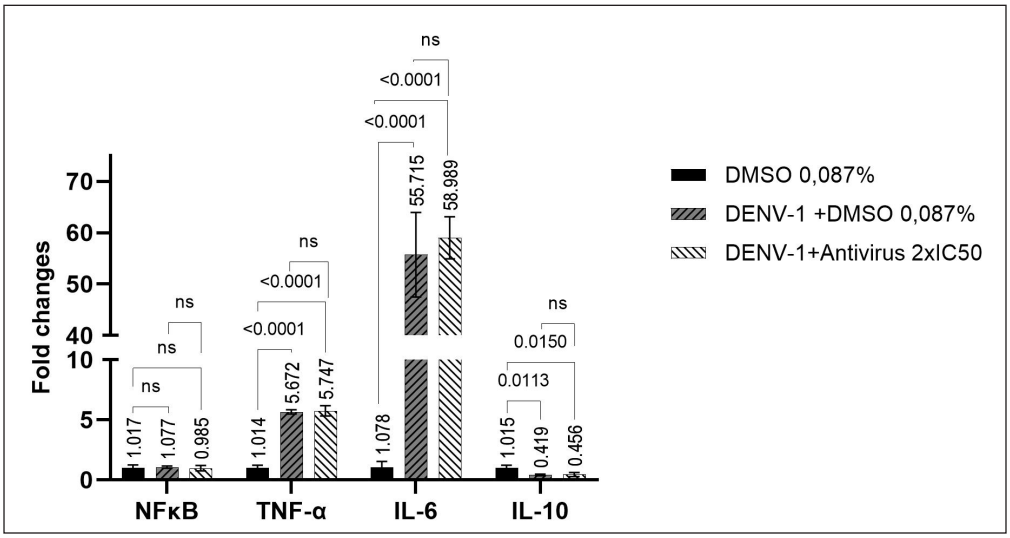


**Figure 2.** The expression of NFκB and cytokines in 24 hours post-treatment butanol fraction of *C. alata* leaf extract 2 × $IC_{50.}$ Relative mRNA expression was analyzed by the delta delta CT method (ΔΔCT).

(Fig. 2). This shows that the effect of the butanol fraction of *C. alata* leaf extract was more observed at 2 hours post-treatment compared to 24 hours post-treatment.

Based on the results, it has been observed that the levels of TNF-α and IL-6 in the group infected with DENV-1 (with or without *C. alata* treatment) were elevated at 24 hours post-treatment compared to 2 hours post-treatment. In contrast, the expression of IL-10 decreased at 24 hours post-treatment relative to 2 hours post-treatment. The expression of NFκB remained relatively constant between 2 hours and 24 hours post-treatment (Figs. 1 and 2).

## DISCUSSION

This research studied the anti-inflammatory activity of the butanol fraction of *C. alata* leaves on DENV-1 infection *in vitro*. The *C. alata* leaf extract itself has been widely studied in previous studies and shows that the active compounds in *C. alata* leaves have DENV antiviral activity both *in vitro* and *in vivo* [23–25,36]. From prior experiments (data not shown), it was found that the total flavonoid content in the butanol fraction of *C. alata* leaves was 31.34%, which is higher compared to the total flavonoid content in the crude or ethanol extracts of *C. alata*, which was 6.82%. From $IC_{50}$ and $CC_{50}$, the SI value of the butanol fraction of *C. alata* leaves was 18.67. The SI is usually used as a pre-clinical screening strategy by determining the toxic and effective therapeutic concentrations ratio. A higher SI value is an early indicator of drug efficacy [37], but the value cannot be extrapolated to doses administered *in vivo* [38]. However, the SI value is a relevant indicator to use in screening procedures for further evaluation of drug candidates [37]. In the dose dependent test ($CC_{50}$) and toxicity test ($IC_{50}$), the cells used were Vero E6 cells. Vero E6 cells are a continuous cell line in the form of epithelial cells obtained from the kidneys of African green monkeys (*Cercopithecus aethiops*) which are extensively used in virology research [39], for example, to evaluate the activity of anti-DENV compounds [40,41] and to produce viruses in the development of DENV vaccines [42]. One of the key limitations of this study is that the $IC_{50}$ value was derived from Vero cells and used as a reference to assess the anti-inflammatory effects in PBMCs. This approach was adopted because conducting dose-dependent assays directly on PBMCs presents challenges, primarily due to the fact that PBMCs are not a continuous cell line, making them more difficult to work with compared to Vero cells.

DMSO at a concentration of 0.087% was used as a negative control because the 2 × $IC_{50}$ butanol fraction of *C. alata* leaves extract contained 0.087% DMSO. Employing DMSO as a negative control helped to minimize bias in the data collected. Administration of the butanol fraction of *C. alata* leaf extract at 2 × $IC_{50}$ resulted in a downregulation at 2 hours post-treatment of NFκB expression and TNF-α, IL-6, and IL-10, but not statistically significant. A study with α-mangostin extract showed the suppression of mRNA TNF-α, IL-6, and IL-10 expression by a dose-dependent manner [32]. In this study, we used one dose, i.e., 2 × $IC_{50}$, and 1 time addition, therefore it is still necessary to explore the optimal dose and time of addition. While this dose was selected to provide initial insights, it may not capture the full range of the compound's effects. Future studies could benefit from using a more robust experimental design, incorporating multiple doses or higher concentrations. This approach would allow for a more comprehensive understanding of dose-dependent effects and potentially yield more pronounced and reliable results.

In this study, we found that the RNA expression of NFκB after treatment with *C. alata* were slightly changed, while the expression of TNF-α and IL-6 was decreased at 2-hours post treatment. *Cassia alata* contain 5'-Tetrahidrox-flavone, Daturametelin H and kaempferol-3,7-diglukoside [23]. This study is concomitant with a previous study that demonstrated that kaempferol inhibits the expression of cytokines induced by NFκB and AP-1 in human umbilical vein endothelial cells (HUVECs) [43]. Flavonoids are also known to inhibit IκBα phosphorylation and degradation and NFκB p65 translocation in HEK 293 cells [44].

In this study, the expression of anti-inflammatory cytokines IL-10 was decreased, meanwhile, the ratio between TNF-α and IL-6 towards IL-10 in *C. alata* treatment group was slightly lower than in the untreated group (data not shown). The ability of *C. alata* and kaempferol as anti-inflammatory agents that can suppress TNF-α expression in DENV infection has not been reported, but several previous studies have shown that kaempferol can significantly inhibit TNF-α expression in aged rat gingival tissues by attenuating NFκB nuclear binding activity [45]. Other studies also show that kaempferol at a concentration of 30 μM can significantly decrease the TNF-α expression and secretion of RAW-264.7 macrophages [46]. Kaempferol diminished the overproduction of TNF-α, IL-1β, and IL-6 in inflammation induced by the H9N2 influenza virus both in *vivo* and in *vitro* by inhibiting several pathways such as TLR4, NF-κB p65 DNA binding activity, MyD88, and MAPKs [47].

In this study, we evaluate the expression of NFκB, TNF-α, IL-6, and IL-10 in 2 time points (2-hours and 24-hours post-treatment based on the kinetic of NFκB; NFκB function as transcription factor so we expect that the effect of *C. alata* will be better observed in early phase of stimulation. The results showed that the effect of *C. alata* leaves butanol fraction was stronger in downregulating the expression of TNF-α, IL-6, and IL-10 at 2 hours post treatment.

In blood vessels, IL-6 can directly or indirectly affect vascular endothelial cells. IL-6 is able to increase the disassembly of VE-cadherin through the induction of VEGF and the expression of the C5a receptor, which causes blood vessel permeability. IL-6 directly stimulates vascular endothelial cells to produce proinflammatory cytokines through trans-signaling [48]. With its ability to stimulate pro-inflammatory cytokines, IL-6 is very influential in plasma leakage. The decrease in IL-6 expression by the butanol fraction of *C. alata* 2 × $IC_{50}$ is very useful in preventing the severity of DENV infection. On the other hand, with the nature of the butanol fraction of *C. alata* which is nontoxic, has anti-DENV activity with a low $IC_{50}$ value, and shows anti-inflammatory activity by reducing IL-6 very significantly, the butanol fraction of *C. alata* has potential as an anti-inflammatory candidate in the future.

Further study is needed to evaluate the potential and mechanism of *C. alata* as anti-inflammation. In another hand, this study reflects the immune response of only one individual, as the PBMC donor was a single person. Future research should involve

more subjects for a better understanding of the immune response across multiple individuals and to obtain more reliable data.

## CONCLUSION

These results suggest the butanol fraction of *C. alata* leaf extract has more pronounced effects at 2 hours post-treatment compared to 24 hours. Butanol fraction of *C. alata* leaf extract potentially used as an antiviral agent with other advantages of potential anti-inflammation effect.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

The anti-inflammatory test used PBMC from a donor who was not being infected with DENV. The study protocol was approved by the Health Research Ethics Committee, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia, Indonesia (Approval No.: KET-1449/UN2.F1/ETIK/PPM.00.02/2023).

## DATA AVAILABILITY

The data presented in this study are available in this article.

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## USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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