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Investigation of the antiviral activity of *Carica papaya* L. leaf extract against dengue virus serotype 2 (DENV-2) in Vero cells

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ABSTRACT: Indonesia carries one of the highest dengue burdens in ASEAN, where treatment remains largely supportive rather than antiviral, motivating the search for antiviral candidates from natural sources such as papaya. Papaya leaves contain secondary metabolites reported to have antiviral properties. This study aimed to evaluated the *in vitro* antiviral activity of 96% ethanol leaf extract against dengue virus serotype 2 and its cytotoxicity. Antiviral activity was tested in Vero cells using a post-infection method, and cytotoxicity was assessed in the same cell line. Phytochemical screening detected alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins. Across four concentrations (125-1000 µg/mL, n = 3 each concentrations) regression gave a fifty percent cytotoxic concentration of 1712.74 µg/mL however, viability did not decline in a dose-dependent manner (R^2 = 0.08), so this value is a rough extrapolation rather than a reliable toxicity threshold. Antiviral testing across three concentrations (25-100 µg/mL, n = 3 each concentrations) did not reach fifty percent inhibition of the cytopathic effect within the tested range, the effective concentration was therefore reported as greater than 100 µg/mL, giving a selectivity index below 9.08. These findings indicate limited antiviral activity against dengue virus serotype 2 under the tested conditions and support further work using wider concentration ranges and fractionation of its bioactive constituents.

KEYWORDS: Antiviral; *Carica papaya* L.; cytotoxicity; DENV-2; Vero cells; phytochemical.

▪ INTRODUCTION

Dengue or dengue fever is a disease caused by a viral infection transmitted through mosquitoes, which is most often found through the female *Aedes aegypti* mosquito, but there are still several mosquitoes that can also be vectors of dengue fever, such as the *Aedes polynesiensis*, *Aedes scutellaris*, and *Aedes albopictus* [1],[2]. Dengue has one of the highest transmission rates in the world, particularly in tropical and subtropical regions [3]. Based on data from Badan Pusat Statistik [4], the morbidity rate for dengue fever reached 57.02 per 100,000 population. This value shows that dengue fever is still a significant health problem in Indonesia. Two decades of national surveillance data show that the dengue incidence rate reached a record high of 91.93 per 100,000 population in 2024 [5] and Indonesia has been reported as the ASEAN country with the highest number of dengue cases [6]. The incidence of dengue fever often rises with climate change, particularly when accompanied by increased rainfall intensity, because such conditions favor the breeding of dengue vector mosquitoes [2]. There are four different serotypes of the dengue virus: serotype 1 (DENV-1), serotype 2 (DENV-2), serotype 3 (DENV-3), and serotype 4 (DENV-4). [7]. Of the four dengue virus serotypes, dengue virus serotype 2 (DENV-2) is the serotype that is often associated with the risk of severe dengue fever, as it has been shown to be significantly linked to severe manifestations such as plasma leakage, hemorrhagic rash, and increased transaminase [8]. Until now, no specific treatment is available for dengue fever [9]. Management remains largely supportive, aimed at reducing disease severity and complications [10]. Therefore, exploration of antiviral candidates against the dengue virus is urgently needed. This exploration is directed at identifying

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a virus-targeted antiviral agent, not at improving supportive care itself, because current management only addresses symptoms and complications rather than the virus, the absence of any clinically approved antiviral is precisely the gap that drives the search for candidate compounds capable of directly inhibiting DENV replication [11].

The use of products based on natural ingredients is receiving a lot of attention from the public. Many studies utilize herbal plants that contain secondary metabolite compounds to develop drugs for dengue fever [12]. Secondary metabolite compounds are organic compounds produced through secondary metabolism. They are not universally present across all living organisms, but they can influence the activity of specific organs, tissues, and cells, so they are widely used as a raw materials for medicines [13]. According to Adeosun & Loots [14], several classes of secondary metabolites, including alkaloids, terpenoids and phenolics are secondary metabolite compounds that can be used as antiviral agents. These compounds are known to combat viruses though several mechanisms, including inhibiting viral entry into host cells, suppressing viral replication, and protein synthesis, modulating host immune responses, and cellular signaling pathways, and exerting direct virucidal activity against virus particles [14]. In recent years, exploration of natural ingredient-based antiviral agents has increasingly developed in line with increasing public attention to the use of biological resources in the development of natural medicines and therapies.

One plant that is frequently used to cure dengue fever is the papaya plant (*Carica papaya* L.). In the leaves of the papaya plant there are various substances that have many benefits, such as vitamin A, vitamin B, vitamin C, the papain enzyme [15]. Meanwhile, the secondary metabolite content contained in papaya leaves includes alkaloids, flavonoids, saponins and tannins [16]. The flavonoid contained in papaya leaves is a type of quercetin flavonoid which is believed to increase the number of platelets in dengue fever sufferers [15]. In research, papaya leaf extract is known to have antiviral activity against the dengue virus because papaya leaves contain flavonoid compounds, quercetin, kaempferol, and chlorogenic acid. This compound is thought to be able to inhibit dengue virus replication by interacting with viral proteins such as NS3 and NS5 proteins which have a role in the process of multiplying viral genetic material in cells [17]. NS3 and NS5 are indispensable components of the DENV replication complex, NS3 functions as a protease/helicase that processes the viral polyprotein and unwinds RNA secondary structures during replication, while NS5 functions as the RNA-dependent RNA polymerase (RdRp) responsible for synthesizing new viral RNA strands [18].

Although Carica papaya leaf extracts and their isolated flavonoids (such as quercetin) have been broadly studied against dengue viruses across various cellular models, critical empirical gaps remain. Prior studies have predominantly focused on prophylactic efficacy or broad viral screening without establishing post-infection therapeutic parameters. Specifically, comprehensive evaluations that simultaneously integrate qualitative phytochemical profiling, Vero cell cytotoxicity testing, and post-infection anti-DENV-2 activity for a 96% ethanol extract batch are currently lacking in the literature. Furthermore, definitive baseline values for 50% cytotoxic concentration (CC_{50}), half-maximal effective concentration (EC_{50}), and the resulting Selectivity Index (SI) for this specific extract batch have not yet been established in standard non-human primate substrates such as Vero cells (*Chlorocebus sabaeus*) [19].

To address these gaps, this study introduces a clinically relevant evaluation framework by utilizing a post-infection treatment protocol on DENV-2-infected Vero cells using a standardized 96% ethanol macerate of C. papaya leaves. Unlike traditional pre-infection testing that models prophylactic action, this post-infection approach specifically targets the intracellular viral replication process after host cell entry. This methodology offers significant novelty, as it directly mirrors real-world clinical scenarios, where therapeutic interventions are administered after the onset of infection rather than prior to viral exposure. Accordingly, the objective of this study was to evaluate the in vitro antiviral activity of a 96% ethanol C. papaya leaf extract against DENV-2 in Vero cells using a post-infection intervention model. Additionally, this research aimed to perform comprehensive phytochemical screening and quantitatively determine the extract's cytotoxicity (CC_{50}), antiviral potency (EC_{50}), and overall safety margin (SI) to establish its potential as a post-infection anti-dengue therapeutic candidate.

▪ MATERIALS AND METHODS

Materials

Papaya leaves (*Carica papaya* L.) were obtained from Jl. Pesarean, Dusun Patung, Pungging Village, District of Pungging, Mojokerto Regency, East Java, Indonesia, with a geographical position of 7°31'27.9"S112 °34'46.0"E. Ingredients used in this study included 96% ethanol (Merck, Germany), Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Ca. No D6429, Germany), Fetal Bovine Serum (FBS) (ExCell Bio, China), dimethyl sulfoxide (DMSO) (Merck, Germany), 0.25% trypsin EDTA (gibco, USA), MTT reagent, Phosphate Buffered Saline (PBS) (Sigma-Aldrich, United Kingdom), and ATP substrate (Promega, USA). Materials used for phytochemical screening include Mayer reagent (Merck, Germany), Wagner reagent (Merck, Germany), Dragendorff reagent (Merck, Germany), chloroform (Merck, Germany), ammonia (Merck, Germany), sulfuric acid (Merck, Germany), 70% ethanol (Merck, Germany), magnesium powder (Merck, Germany), hydrochloric acid (HCl) (Merck, Germany), Liebermann-Burchard reagent (Merck, Germany), ferric chloride ($FeCl_3$) 1% (Merck, Germany). This research also used Vero cells (ATCC CCL-81TM, USA) and dengue virus serotype 2 (DENV-2) isolates from Surabaya with GenBank accession number KT012509.

Methods

Papaya leaf extraction

Papaya leaves are washed thoroughly using water to remove dirt and other contaminants, then the papaya leaves are cut into small pieces and dried in the sun until completely dry. Next, the dried papaya leaves are mashed using a blender with the aim of increasing the surface area so that the diffusion process of secondary metabolite compounds becomes faster [20].

A ratio of 1:10 (w/v) was used to macerate 100 g of papaya leaf powder in 1000 mL of 96% ethanol. In an airtight glass container, maceration was done for three days at room temperature. After the maceration process was completed, the filtrate is separated from the residue using filter paper, then the evaporation process is carried out on the filtrate resulting from the maceration. Conventional evaporation was done at room temperature until a thick extract was produced. Next, the yield value is computed using the formula found in Equation 1.

$$yield\ (\%) = \frac{Extract\ weight}{Dry\ leaf\ weight} \times 100\% \qquad \text{(Eq.1)}$$

Phytochemical screening

Phytochemical screening was carried out using qualitative methods to identify secondary metabolite content in papaya leaf extract. The tests conducted included testing for alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins. No reference-standard positive controls (e.g., quercetin for the flavonoid test or tannic acid for the tannin test) were tested in parallel with the samples; positive identifications were based on characteristic color changes and precipitates, as defined for each qualitative reaction, in accordance with the standard qualitative phytochemical screening protocol [21],[22].

Alkaloid test

The Mayer, Wagner, and Dragendorff reagents were used in the alkaloid test. A test tube was filled with 1 mL of papaya leaf extract, 1 mL of ammonia, and 1 mL of chloroform. The solution was then heated in a water bath and shaken; the mixture was separated into three equal-volume test tubes. In the three test tubes, 3 drops of 2N sulfuric acid were added, then added with each reagent used. The first tube was added with Mayer's reagent. The second tube was filled with Wagner's reagent. Dragendorff's reagent was added to the third tube. Positive results are shown by the creation of an orange precipitate for Mayer's reagent, a brown precipitate for Wagner's reagent, and a white precipitate for Dragendorff's [23].

Flavonoid test

The flavonoid test involved adding 3 mL of 70% ethanol to a test tube containing 1 mL of papaya leaf extract solution, shaking the tube, and heating it. Two drops of concentrated HCl and 0.1 g of magnesium powder were added after mixing. Positive outcomes are indicated by the production of a yellow solution [21].

Saponin test

In order to perform the saponin test, 1 mL of papaya leaf extract solution was combined with 10 mL of distilled water, and the mixture was then brought to a boil in a water bath. The mixture was then vigorously mixed until froth developed, and it was left for fifteen minutes. The extract is deemed to contain saponin if the foam lasts for this long [23].

Steroid test

The steroid test was carried out by adding Liebermann-Burchard reagent (2 mL of sulfuric acid and 2 mL of anhydrous acetic acid) and 3 mL 70% ethanol to 1 mL papaya leaf extract solution. The creation of a green or blue solution indicates a positive result [23].

Triterpenoid test

To perform the triterpenoid test, 1 mL of papaya leaf extract was mixed with 3 mL of sulfuric acid and 3 mL of chloroform. The creation of a brownish-red solution indicates positive results [23].

Tannin test

The tannin test was performed by heating 1 mL of papaya leaf extract solution in a water bath and adding 2-3 drops of 1% $FeCl_3$. The creation of a greenish-brown solution indicates positive results [23].

Cytotoxicity assay

Vero cells derived from the kidneys of African green monkeys were used in this research. Vero cells were maintained and propagated in DMEM containing 10% FBS. Vero cells with a density of 5×10^4 cells/mL which had been previously cultured were added with 2 mL 0.25% trypsin EDTA which functioned to release Vero cells from the flask where they were propagated [24]. Then, the flask was incubated for 5 minutes in a CO_2 incubator until the cells became monolayer. Add 2 mL of media (DMEM and FBS) into the flask, homogenize slowly using a pipette, then add 100 µL of the media containing the Vero cells into the 96-well assay plate in each well. Incubate for 24 hours [25]. After that, the morphology of the Vero cells was observed using an inverted microscope, then absorbance readings were carried out using a microplate reader.

The thick extract obtained from maceration was weighed and dissolved using 0.1% DMSO, the mixture was then vortexed for 2 hours until homogeneous. The preparation of a test solution consisting of a stock solution and a working solution is obtained from the dilution calculation as in Eq 2.

$$M_1 \times V_1 = M_2 \times V_2 \quad \text{(Eq. 2)}$$

The stock solution obtained was then diluted using culture media (DMEM and FBS) to make working solutions with several concentrations, namely 1000; 500; 250; and 125 µg/mL. The working solution was then added as much as 100 µL to the Vero cells that had been prepared in the plate. Each working solution concentration was repeated 3 times to ensure the accuracy of the results. The plate was then incubated for 24 hours in a CO_2 incubator.

After incubation was complete, the media in each well was discarded, then the cells were washed using PBS to remove remaining media. After that, fill each well with 20 µL of MTT solution and incubate once again for 4 hours until formazan crystals form [26]. The crystals formed were then dissolved using DMSO solution. After that, absorbance was determined using a microplate reader with a 595 nm wavelength. The cytotoxic concentration 50 (CC_{50}) value is derived using the regression equation that is obtained after the percentage of cell viability is determined by comparing the absorbance of the treatment to control cells [27].

Antiviral assay

Vero cells derived from the kidneys of African green monkeys were used in this research. Vero cells were maintained and propagated in DMEM containing 10% FBS. Vero cells with a density of 5×10^4 cells/mL which had been previously cultured were added with 2 mL 0.25% trypsin EDTA which functioned to release Vero cells from the flask where they were propagated [24]. Then, the flask was incubated for 5 minutes in a CO_2 incubator until the cells became monolayer. Add 2 mL of media (DMEM and FBS) into the flask, homogenize slowly using a pipette, then add 100 µL of the media containing the Vero cells into the 96-well assay plate in each well. Incubate for 24 hours [25]. After that, the morphology of the Vero cells was observed using an inverted microscope, then absorbance readings were carried out using a microplate reader.

The thick extract obtained from maceration was weighed and dissolved using 0.1% DMSO, the mixture was then vortexed for 2 hours until homogeneous. The preparation of a test solution consisting of a stock solution and a working solution is obtained from the dilution calculation as in Eq 2. The stock solution obtained was then diluted using culture media (DMEM and FBS) to make working solutions with several concentrations, namely 100; 50; and 25 µg/mL.

Antiviral activity against DENV-2 was tested using the post-infection method on Vero cells. Vero cells that had been prepared were first infected using DENV-2 virus at a concentration of 2×10^3 FFU/mL. The use of focus-forming units (FFU) indicates that virus titration followed a focus-forming assay (FFA) rather than a plaque assay, an approach commonly used for flaviviruses such as DENV-2 in Vero cells because it is faster and does not require an agarose overlay [28]. Then, 100 µL of DENV-2 virus were inserted into each well and then incubated for 1 hour at 37°C in a 5% CO_2 incubator for the virus infection process. Vero cells were seeded at 100 µL/well of a 5×10^4 cells/mL suspension, following the same Vero cell preparation in the Cytotoxicity section. Based on this, the multiplicity of infection (MOI) was approximately 0.04. After the infection process was completed, extract papaya leaves with a concentration of 100; 50; and 25 µg/mL were added as much as 100 µL to infected cells and incubated again for 48 hours in a CO_2 incubator at 37°C. A cell control and a virus control were included in each assay run, a control design comparable to that used in other *in vitro* plant-extract antiviral screening studies against arboviruses [29]. No drug-based positive control e.g., ribavirin or chloroquine was included in the antiviral assay, the absence of a positive control limits the ability to validate the assay system against a molecule of known antiviral activity, and this is acknowledged as a limitation in the discussion.

Antiviral activity was evaluated using an ATP-based luminescence method. ATP substrate was added to the wells as much as 100 µL, then the luminescence intensity was measured using a GloMax Luminescence microplate reader instrument (Promega, USA). The 50% effective concentration (EC_{50}) value is calculated based on the percentage of virus inhibition against the control [30]. Meanwhile, the selectivity index (SI) is calculated based on a comparison between the CC_{50} and EC_{50} values [31].

Data analysis

Cytotoxicity test data were obtained from absorbance measurements using the MTT method. The cell viability percentage was calculated using the formula in Equation 3.

$$Cell\ viability\ (\%) = \frac{Sample\ absorbance\ - media\ control}{cell\ control\ - media\ control} \times 100 \qquad \text{(Eq. 3)}$$

The obtained cell viability values were then plotted against the extract concentration to generate a curve representing the relationship between extract concentration and cell viability. CC_{50} values were determined through linear regression analysis. For each replication, CC_{50} values were calculated and then presented as the mean and standard deviation. Data analysis and visualization were performed using Microsoft Excel 2021.

Antiviral activity test data were obtained from the percentage of DENV-2 inhibition at various extract concentrations (%CPE). The percentage of CPE can be calculated using the formula in Eq 4.

$$\%CPE\ = \frac{Sample\ absorbance\ - media\ control}{cell\ control\ - media\ control} \times 100 \qquad \text{(Eq. 4)}$$

The obtained CPE percentage values were then plotted against the extract concentration to generate a curve showing the relationship between extract concentration and CPE percentage. The EC_{50} value was determined through linear regression analysis. The SI value was calculated by comparing the CC_{50} and EC_{50} values. Data analysis and visualization were performed using Microsoft Excel 2021.

RESULTS

Extraction yield

Maceration of 100 g papaya leaf powder in 96% ethanol yielded a thick extract corresponding to an extraction yield of 6.40%, calculated using Eq.1. This value is markedly lower than the 15.66% yield reported by Abdel-Halim *et al.* using conventional static maceration of papaya leaves using 80% aqueous ethanol at room temperature [32]. Since both studies used the same extraction principle on the same plant part, the difference is more likely attributable to protocol details than to the extraction technique itself. Specifically, the higher ethanol concentration used here 96% vs 80%, which is less effective at extracting polar compounds, and the sun-drying of the raw leaves prior to maceration, which offers no control over temperature and may cause uneven moisture removal or degradation of heat sensitive metabolites compared with oven drying.

Phytochemical screening of papaya leaf extract

Phytochemical screening was carried out to identify the secondary metabolite content in papaya leaf extract (*Carica papaya* L.). The results of phytochemical screening of papaya leaf extract show that papaya leaves have several secondary metabolite compounds, namely alkaloids, flavonoids, saponins, steroids, triterpenoids and tannins.

Table 1. Phytochemical screening results of papaya leaf extract.

Compound group	Reagent	Observation result	Intensity
Alkaloid	Mayer reagent	Orange precipitate	+
	Wagner reagent	Reddish-brown precipitate	++
	Dragendorff reagent	White precipitate	+++
Flavonoid	Mg + concentrated HCL + ethanol	Yellowish-green solution	++
Saponin	Foam test	Stable form formed	+
Steroid	Liebermann-Burchard reagent	Green solution	++
Triterpenoid	Chloroform+ concentrated sulfuric acid	Brown solution	+
Tannin	$FeCl_3$ 1%	Greenish-brown solution	++

Cytotoxicity assay on Vero cells

The MTT assay method was used to investigate the cytotoxicity of papaya leaf extract against Vero cells. Papaya leaf extract has concentrations of 125, 250, 500, and 1000 µg/mL. Vero cell viability values are obtained from absorbance readings using a microplate reader, after which the absorbance value data will be processed to produce graphs and regression equations.

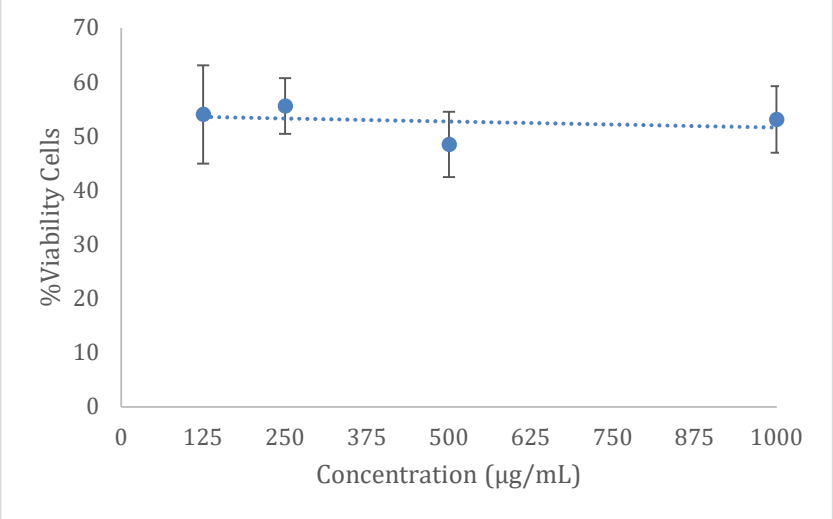

Figure 1. Viability of Vero cells treated with papaya leaf extract at various concentrations.

Based on results of cytotoxicity tests on Vero cells, viability across the concentrations tested did not show a clear dose-dependent decrease. Vero cell viability percentage at concentration 125; 250; 500; 1000 µg/mL averaged across 3 replicates, was 54.01±9.09%, 55.56±5.16%, 48.46±6.02%, and 53.09±6.17%, respectively. From these data, a regression equation was obtained with a value of R^2 = 0.08, indicating a poor linear fit and no clear dose-response relationship within the tested range. The CC_{50} value obtained from the regression equation was 1712.74 µg/mL. At each concentration, 3 replications were carried out. The CC_{50} value exceeds the highest concentration directly tested (1000 µg/mL) and should therefore be treated as an extrapolated preliminary estimate.

Morphological observation of Vero cells

Microscopic observations were carried out on Vero cells without treatment (control) and Vero cells that had been treated with papaya leaf extract at a concentration of 1000 µg/mL.

(a) (b)

Figure 2. Morphological appearance of Vero cells observed under inverted microscope magnification 4× (a) untreated Vero cells (control); (b) Vero cells treated with papaya leaf extract concentration 1000 µg/mL.

In Figure 2. (a) You can see the control Vero cells showing a dense monolayer and attached to the surface of the plate. Meanwhile, in Figure 2. (b) You can see a decrease in density in the Vero cells and some cells appear to have changed their shape to become rounder compared to the control Vero cells.

Antiviral activity against DENV-2

Papaya leaf extract's antiviral properties against dengue virus serotype-2 (DENV-2) were evaluated in Vero cells using the post-infection method. This test was carried out at a concentration of 25, 50, and 100 µg/mL. Based on the results of antiviral activity testing against the DENV-2 virus, the cytopathic effect (CPE) value decreased as the concentration of papaya leaf extract increased.

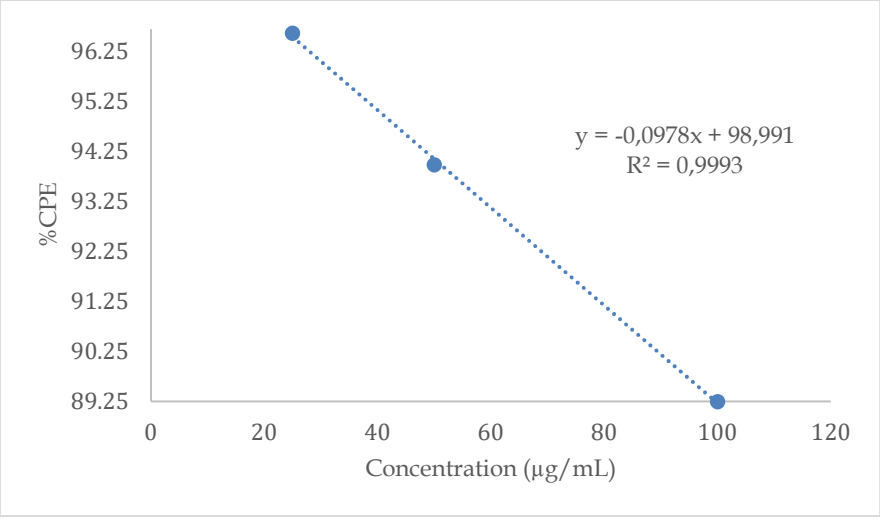

Figure 3. Relationship between papaya leaf extract concentration and %CPE

The EC_{50} value of papaya leaf extract against DENV-2 obtained from processing the regression equation data in Figure 3; based on this regression, 50% inhibition of the cytopathic effect was not reached within the tested concentration range (25-100 µg/mL), because the maximum tested concentration 100 µg/mL did not achieve 50% inhibition of the cytopathic effect, the EC_{50} value is reported as EC_{50} >100 µg/mL rather than as a numerically extrapolated value. Accordingly, the selectivity index (SI), calculated as the quotient of CC_{50} and EC_{50} is reported as SI < 9,08 rather than as a single numerical value.

DISCUSSION

Phytochemical screening of papaya leaf extract revealed that the extract contains secondary metabolites, namely alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins. According to research from Alomair *et al.* [33], shows that flavonoids can interact with nonstructural proteins, namely NS3 and NS5, which play a role in the virus replication process. Apart from that, research from Faisal *et al.* [34] also showed that alkaloids have antiviral activity, alkaloids can inhibit DENV-2 virus protein synthesis. However, the presence of these secondary metabolite compounds cannot directly indicate high antiviral activity, because biological activity is influenced by the levels of active compounds, compound stability, and the overall composition of the extract [35]. It should be emphasized that these NS3/NS5 binding and protein-synthesis-inhibition mechanisms were proposed based on *in silico* modeling or other experimental systems, rather than demonstrated directly in the present study, the specific molecular interactions between papaya leaf extract compounds and DENV-2 targets therefore remain to be experimentally confirmed in this system [17]. It should be noted that the qualitative phytochemical tests were interpreted from the expected color and precipitate reactions without parallel testing of reference-standard positive controls for each compound class [22]. Although the observed reactions matched the characteristic changes expected for each group, in the absence of reference standards the possibility of false-positive results from interfering compounds in the crude extract cannot be entirely excluded. Confirmatory quantitative methods (e.g., TLC or HPLC with authentic reference standards) are recommended in future work to further validate these qualitative screening results.

Papaya leaf extract positively contains secondary metabolite compounds like alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins, according to research by Kurniadi *et al.* [36]. This is consistent with the findings of the phytochemical screening of papaya leaf extracts. Papaya leaf extract, however, contains secondary metabolite chemicals including alkaloids, flavonoids, saponins, tannins, terpenoids, and phenolics, according to research findings from Yana *et al.* [21]. Different from the others, research from Waruwu *et al.* [37] said that papaya leaf extract obtained from the highlands contains secondary metabolite compounds flavonoids, tannins, terpenoids and phenolics, while papaya leaf extract obtained from the lowlands only contains terpenoids.

The results of the cytotoxicity test on papaya leaf extract showed a CC_{50} value of 1712.74 µg/mL. The CC_{50} value is the concentration of a compound that causes death or a 50% reduction in viability of the cells used in the test. The CC_{50} value is used to describe the level of toxicity of a material to its host cells. The higher the CC_{50} value produced, the lower the toxic properties of the compound to cells [27]. Based on Indrayanto's classification [27], extracts are categorized as highly toxic (CC_{50} ≤ 20 µg/mL), moderately toxic (CC_{50} 21-200 µg/mL), weakly toxic (CC_{50} 201-500 µg/mL), or non-toxic (CC_{50} above 500 µg/mL). In this study, the CC_{50} value of 1712.74 µg/mL would nominally place it in this low-toxicity range. This threshold-based framing, however, applies most directly to a well-fitted regression. The CC_{50} value comes from a regression with R^2 = 0.08, meaning the four averaged viability does not decrease monotonically with concentration, and the apparent decrease between the first and last point is smaller than the point-to-point fluctuation. Consequently, describing papaya leaf extract as having low toxicity based on this CC_{50} value overstates what the data support, the more accurate statement is that no clear cytotoxic dose-response was observed for Vero cells within the 125-1000 µg/mL range tested, and the regression-derived CC_{50} should be treated as a rough, low confidence extrapolation rather than a reliable toxicity threshold.

It should be noted that the standard deviation of the CC_{50} value was larger than the CC_{50} estimate itself, reflecting substantial variability among the three replicates. Part of this variability may relate to known interference of plant extracts with the MTT-formazan reaction, which can distort apparent viability readings [38]. This high variability limits the precision of reported CC_{50} value and should be considered a limitation when interpreting the cytotoxicity results.

The microscopic observations of Vero cells in Figure 2 further show that Vero cells without extract treatment form a dense monolayer and adhere to the surface of the plate. Meanwhile, Vero cells exposed to papaya leaf extract with a concentration of 1000 µg/mL showed a slight decrease in cell density as well as morphological changes in the form of some cells becoming rounder. However, most of the cells still appeared to be attached to the surface of the plate and there was no visible massive monolayer damage.

Morphological changes in the form of cell rounding are an early indicator of stress or disruption to the cell membrane [39]. Previous research, from Lawan [40] shows that morphological changes in Vero cells can be characterized by changes in cell shape, reduced contact between cells, and detachment of cells from the plate to which they are attached. The results of papaya leaf extract with a concentration of 1000 µg/mL still showed mild morphological changes. Thus, the morphological observations are broadly consistent with the finding that the extract did not produce strong, dose-dependent cytotoxicity within the concentrations tested, though as noted above the weak regression fit means this should not be read as confirming a specific, reliable CC_{50} threshold.

The antiviral activity test using the post-infection method produced an EC_{50} value that as noted above, could not be directly measured within the tested range and is reported as EC_{50} > 100 µg/mL. The EC_{50} value indicates the concentration of a compound that is capable of producing 50% of the antiviral effect on the target being tested. The stronger the antiviral activity produced, the smaller the EC_{50} value obtained [41]. Based on Martha's classification, EC_{50} compounds are categorized as very strong (EC50 < 50 µg/mL), strong (50-100 µg/mL), moderate (101-150 µg/mL), weak (151-250 µg/mL), or inactive (EC50 above 250 µg/mL) [42]. The present extract falls into this inactive category, although the exact EC_{50} could not be determined within the concentration range tested here, the fact that 50% inhibition was not reached even at the highest tested concentration already indicates that papaya leaf extract did not show strong antiviral activity against DENV-2 under these test conditions. From the CC_{50} and EC_{50} results that were obtained, the resulting SI value is reported as SI < 9.08 rather than as a single precise value, since it is derived from EC_{50} > 100 µg/mL bound. Based on Indrayanto's research [27] compounds with SI values above 2 sometimes used as a minimal cutoff, because the SI value obtained here is only an upper bound rather than a precisely determined value, whether papaya leaf extract falls below this threshold cannot be confirmed without testing at higher antiviral concentrations that directly bracket the 50% inhibitory response.

It is also important to note that the CC_{50} and EC_{50} assessments were based on only four and three tested concentrations, respectively. The CC_{50} value exceeds the highest concentration 1000 µg/mL by an even wider margin than previously reported, making it a more substantial extrapolation rather than a close approximation, while the EC_{50} could not be reached within the tested range 25-100 µg/mL and is accordingly reported as EC_{50} > 100 µg/mL rather than as an extrapolated numerical value. Reliable determination of EC_{50}/CC_{50} generally requires several concentrations bracketing the response on both sides of the 50% point [41]. Consequently, the SI value reported here is only a conservative bound rather than a precisely calculated estimate. Future studies should test a wider and more finely spaced range of concentrations, particularly above 100 µg/mL for the antiviral assay and above 1000 µg/mL for the cytotoxicity assay, so that the EC_{50} and CC_{50} can be directly measured rather than reported as bounds.

The low antiviral activity obtained can be influenced by several factors. The content of secondary metabolites in plants depends on the growing location, environmental conditions, age of the plant, and the plant preparation process before testing. These differences can affect the quantity and stability of the active compounds responsible for the biological activities in the extract [35]. Apart from that, the extraction process also affects the quality of the compounds obtained [43]. The open solvent evaporation method allows degradation or oxidation of some sensitive secondary metabolite compounds [22],[44]. This condition can cause a decrease in the biological activity of the extract, including its antiviral activity.

Several previous studies have proven that papaya leaves (*Carica papaya* L.) have antiviral activity against dengue viruses through the content of secondary metabolite compounds in them. Results from Agustina's research shows that papaya leaves contain flavonoid compounds, alkaloids, saponins, and the enzyme papain [45]. It was stated that quercetin, which is a type of flavonoid compound, is thought to be able to inhibit the replication of the DENV-2 virus, while the papain enzyme plays a role in supporting the recovery of the condition of dengue fever sufferers. The flavonoid chemicals in papaya leaves, particularly quercetin, have been reported based on *in silico* modeling rather than direct experimental testing to interact with dengue virus

proteins including NS3 and NS5, which are involved in the virus replication process, according to another study by Alomair *et al.* [33]. Apart from that, research from Faisal *et al.* also reported, based on literature review, that alkaloids may possess antiviral activity through inhibition of viral protein synthesis [34]. It should be noted that these NS3/NS5 interaction and protein synthesis-inhibition mechanisms were proposed *from in silico* or other experimental systems and were not directly demonstrated for papaya leaf extract in the present study. The results of this research show that papaya leaves have been widely studied as a natural ingredient that has biological activity against the DENV-2 virus. However, differences in extraction methods, types of solvents, cell systems, and testing methods can cause differences in the antiviral activity results obtained.

Overall, this study provides initial data regarding the cytotoxicity profile and antiviral activity of papaya leaf extract against the DENV-2 virus using post-infection methods. Even though the extract showed a safe level of toxicity against Vero cells, the antiviral activity obtained was still low. Therefore, further research is needed such as optimizing extraction methods, fractionating active compounds, and testing using other methods (pre-infection and during infection) to obtain more specific and effective antiviral activity against the dengue virus. Taken together, the finding that EC_{50} exceeds 100 µg/mL, placing it at beyond the weak-to-inactive boundary of Martha's classification together with SI value bounded only at below 9.08, indicates that the crude 96% ethanol extract, on its own, has limited potential as a direct antiviral candidate against DENV-2 under the tested conditions [42]. Rather than warranting direct advancement of the raw extract, these findings support fractionation and isolation of its bioactive constituents, particularly the flavonoid fraction, as the more promising next step toward identifying a more potent and selective anti-DENV-2 candidate.

▪ CONCLUSION

This study showed that 96% ethanol extract of *Carica papaya* L. leaves contains several secondary metabolites, namely alkaloids, flavonoids, saponins, steroids, triterpenoids, and tannins. The extract exhibited a CC_{50} value of 1712.74 µg/mL against Vero cells, however, because cell viability did not decline in a clear dose-dependent manner across the concentrations tested (R^2 = 0.08), this value should be regarded as a preliminary, low-confidence estimate rather than a firm toxicity threshold. Using the post-infection method, the extract did not reach 50%inhibition of the DENV-2 cytopathic effect within the tested concentration range (25-100 µg/mL) the EC_{50} was therefore reported as > 100 µg/mL, corresponding to a selectivity index of < 9.08. taken together, these findings indicate that the extract has limited antiviral activity against DENV-2 under the conditions tested.

Future studies should test wider and more closely spaced concentration ranges to allow direct, non-extrapolated determination of the CC_{50} and EC_{50} values. The extraction method should also be optimized, for example through ultrasound-assisted extraction (UAE) or temperature-controlled drying, to improve yield and preserve heat-sensitive secondary metabolites. In addition, fractionation of the extract using column chromatography is recommended to isolate and concentrate its bioactive constituents, and antiviral activity should be evaluated using the pre-infection method to assess whether the extract is more effective as a prophylactic than as a therapeutic agent. Characterization of the extract's active components by LC-MS/MS is further recommended to identify and quantify the specific flavonoid and alkaloid constituents responsible for its biological activity.

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