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Inhibition Test of Cassava Leaves (*Manihot esculenta* Crantz) Flavonoid Nicotiflorin on Replication of Dengue Virus Serotype 1 in Vitro

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ABSTRACT

Background: Dengue fever is a disease caused by the bite of *Aedes aegypti* mosquitoes infected with the dengue virus. Dengue virus infection can lead to hemorrhagic fever and even death. Currently, treatment for dengue infection is supportive, as there are no commercially available antiviral drugs. Nicotiflorin, a compound found in cassava leaves (*Manihot esculenta* Crantz), has shown potential as an antiviral agent against dengue.

Aims: This study aimed to determine the inhibitory effect of the flavonoid nicotiflorin, derived from cassava (*Manihot esculenta* Crantz) leaves, as an antiviral agent against dengue virus serotype 1 (DENV-1) in vitro.

Methods: A post-test-only control group design was utilized in this experimental research, comprising one control group alongside thirteen treatment groups. For the cytotoxicity assessment, the treatment groups were exposed to seven varying concentrations (1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL), while six different concentration levels (1.5 µg/mL, 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL) were employed in the inhibition assay. The control group received 0.2% DMSO as a negative control. Antiviral inhibition was assessed using the Focus Forming Unit (FFU) Assay, while cytotoxicity was evaluated using the Microtiter Tetrazolium Assay (MTT Assay). Statistical analyses included the Shapiro-Wilk test for normality, the Kruskal-Wallis test for hypothesis testing, and post-hoc analysis to assess significant differences among treatment groups.

Results: The CC_{50} and IC_{50} values of nicotiflorin from cassava leaves were determined to be 19.24 µg/mL and 0.9550 µg/mL, respectively, yielding a Selectivity Index (SI) of 20.14. These findings indicate that the flavonoid nicotiflorin from cassava leaves exhibits selective antiviral activity against DENV-1 replication. Statistical analysis revealed a non-normal data distribution ($P < 0.05$), a significant difference among groups based on the Kruskal-Wallis test ($P < 0.05$), and no statistically significant differences among specific concentrations in the post-hoc test.

Conclusion: Nicotiflorin from cassava leaf (*Manihot esculenta* Crantz) has inhibitory activity on the replication of dengue virus serotype 1 strain in vitro.

Keywords: *Manihot esculenta C., Dengue virus, Flavonoid nicotiflorin, Focus Forming Unit Assay, Microtiter Tetrazolium Assay.*
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1. Introduction

Dengue hemorrhagic fever (DHF) is a viral infection spread by the bite of *Aedes aegypti* mosquitoes carrying one of the four dengue virus serotypes: DENV-1, DENV-2, DENV-3, or DENV-4 (Wong et al., 2020). Plasma leakage and potentially fatal outcomes can arise as a consequence of dengue virus infection. As reported by the WHO in 2019, dengue hemorrhagic fever (DHF) is a tropical illness that has long posed a global health challenge, with its incidence increasing 30-fold over the past five decades. It continues to be a major public health concern worldwide, including in Indonesia. The World Health Organization estimates that about 2.5 billion people around 40% of the global population are at high risk of dengue infection. Globally, there are an estimated 400 million cases and 22,000 deaths each year. In Indonesia, DHF was first detected in Surabaya in 1968, and since then, cases have steadily risen, spreading to all provinces (WHO, 2023).

At present, no targeted therapy exists for dengue. The treatment is mainly symptomatic and supportive, aimed at managing plasma leakage caused by increased capillary permeability and bleeding. In addition, misdiagnosis of DHF is still common and often leads to inappropriate treatment. Poor clinical management can increase the risk of morbidity, mortality, medical costs, and the development of antibiotic resistance (Made Susila Utama et al., 2019). Until now, there is still no effective antiviral drug or vaccine available for the treatment or prevention of dengue disease. Although several antiviral candidates have been developed, none have been fully effective (Torres-Flores et al., 2022).

Indonesia has rich biodiversity, including many plants with potential medicinal properties. Cassava (*Manihot esculenta* Crantz), frequently utilized in traditional healing practices, possesses leaves rich in bioactive substances like triterpenoids, flavonoids, and saponins—compounds known for their antiviral and antimicrobial properties. Among these, nicotiflorin stands out as a predominant flavonoid in cassava leaves, exhibiting a range of biological effects such as liver protection, antioxidant activity, anti-inflammatory action, and support for endothelial cell health (Mohidin et al., 2023). Using HPLC-FTICR-MS analysis, the extract was found to contain seven flavonoid constituents, with their respective proportions of the total flavonoid content identified as: clovin (0.96%), myricetin-3-O-rutinoside (4.81%), robinin (1.25%), rutin (58.89%), hyperoside (2.51%), nicotiflorin (29.31%), and narcissin (2.28%). Therefore, cassava leaves (*Manihot esculenta* Crantz) have the potential to be explored as functional food or therapeutic agents to promote human health (Jampa et al., 2022).

Nicotiflorin, also known as Kaempferol 3-O-β-rutinoside, is one of the pure compounds found in various medicinal plants. Previous studies have reported that nicotiflorin is a potent hepatoprotective agent capable of protecting the liver against immunological and chemically induced acute damage, which may be attributed to its antioxidant and immunoregulatory properties. In addition, nicotiflorin exhibits anti-inflammatory activity, provides endothelial protection, and can reduce nitric oxide (NO) release during endothelial cell injury (Rani et al., 2022).

In silico studies suggest that nicotiflorin has potential antiviral activity against SARS-CoV-2 by inhibiting its main protease. Docking simulation results also indicated that nicotiflorin has the potential to function as a suppressor of SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) and 3CLpro enzymes (da Silva et al., 2020). The dengue virus (DENV) NS2B-NS3pro serine protease is a primary drug target for dengue treatment. Utilizing both computational simulations and laboratory experiments, Dhar and Vivek's research highlighted the efficacy of Azadirachta indica-derived bioflavonoids in combating dengue. Their study revealed that kaempferol-3-O-rutinoside (−9.555 kcal/mol), epicatechin (−7.622 kcal/mol), rutin (−9.324 kcal/mol), and hyperoside (−7.879 kcal/mol) exhibited greater inhibitory activity against DENV protease compared to quercetin (−6.94 kcal/mol), as reflected by their stronger binding energies (Dwivedi et al., 2021). In an analysis of kaempferol's effect on viral protein expression, a study conducted by Chit Care et al found that several commonly used loading control proteins, including GAPDH, β-actin, and vinculin, were significantly downregulated following treatment with kaempferol. Due to the presence of internal ribosome entry site (IRES) sequences enabling translation independent of the 5' cap during cellular stress, GRP78 and Hsp70 were chosen for assessment. Kaempferol had no impact on Hsp70 expression, which was therefore employed as a loading control, whereas GRP78 expression was specifically examined alongside it (Care et al., 2020).

Although these studies indicate the potential of nicotiflorin, there has been no research that specifically evaluates the antiviral effect of pure nicotiflorin isolated from cassava leaves against dengue virus replication. Therefore, this study aimed to assess the inhibitory effect of nicotiflorin on the replication of Dengue Virus Serotype 1

(New Guinea C strain) in vitro.

2. Methods

Study design/ Research procedures

This experimental study utilized a posttest-only control group design, employing dengue virus serotype 1 (DENV-1) and Vero cells as experimental models. The study consisted of 13 treatment groups and one control group. The treatment groups were categorized into six concentrations of nicotiflorin extracted from cassava leaves (*Manihot esculenta* Crantz) for the inhibition assay, and seven concentrations for the cytotoxicity assay. A simple random sampling technique was used. The research was carried out at the Clinical Microbiology Laboratory, Department of Microbiology, Faculty of Medicine, Universitas Indonesia, located in Cikini, Central Jakarta, between May and June 2024. The number of replications was determined using Federer's formula, resulting in an *R-value* of 3.5. Inclusion criteria included DENV-1 propagated in a cell culture medium and virus inoculation at a multiplicity of infection (MOI) of 0.2. The exclusion criterion was contamination of the culture medium by microorganisms other than the dengue virus. This study received ethical approval from the Research Ethics Committee of the Faculty of Medicine, Swadaya Gunung Jati University, with approval letter number 11/EC/FKUGJ/IV/2024.

Measurements

a. Materials
In this study, the flavonoid compound nicotiflorin was commercially sourced from MarkHerb Manufacturer, catalog number FLV-1-100. The dengue virus and Vero cells utilized were obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia, with passage numbers 4 and 2, respectively.

b. Determination of cytotoxicity through CC_{50} value
The MTT assay technique was employed in this study to assess the cytotoxicity of the flavonoid compound nicotiflorin on Vero cells by measuring the percentage of cell viability. Cell viability refers to the ability of cells to remain alive and functional. This experiment was evaluated based on optical density (absorbance) readings, with higher absorbance indicating greater cell viability due to more intense color development.
Vero cells were first cultured in 96-well plates, after which they were exposed to various concentrations of nicotiflorin derived from cassava leaves—specifically, 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL. The plates were maintained at 37°C for a 48-hour incubation period. Following this, the culture medium was substituted with an MTT reagent, and the cells underwent an additional 2-hour incubation to facilitate the development of formazan crystals. Absorbance was measured using an ELISA reader at a wavelength of 450 nm. The absorbance values were then analyzed using GraphPad Prism software to determine the half-maximal cytotoxic concentration (CC_{50}) of nicotiflorin.

c. Determination of antiviral activity through IC_{50} value
To assess the suppression of DENV-1 replication, the percentage of viral infectivity was quantified through the focus-forming unit (FFU) method, following treatment with nicotiflorin, a flavonoid isolated from cassava leaves (*Manihot esculenta* C.). In this method, Vero cells infected with DENV-1 exhibit brown foci, which represent sites of viral replication. Various concentrations of nicotiflorin were applied to infected cells to determine its antiviral activity. The resulting FFU data were analyzed using GraphPad Prism software to calculate the half-maximal inhibitory concentration (IC_{50}) value.
In the inhibition experiment, Vero cells were simultaneously exposed to the virus at a multiplicity of infection (MOI) of 0.2 and treated with varying concentrations of nicotiflorin—specifically, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, and 1.5 µg/mL. Following a two-hour incubation period at 37°C, any unattached viral particles were eliminated by rinsing the cells with 200 µL of DMEM. Following a 72-hour incubation period at 37°C, the culture supernatants were harvested for viral quantification, having previously exposed the cells to treatment.

d. Selectivity index
Selectivity index of nicotiflorin was analyzed with the formula CC_{50}/IC_{50}.

Statistical techniques

Statistical analysis of the collected data was carried out with the help of computer-based software. Given that the sample size was under 50, the Shapiro-Wilk method was utilized to evaluate data normality. For hypothesis evaluation, the Kruskal-Wallis nonparametric test was employed. Following this, a post hoc analysis was conducted to ascertain whether significant differences existed between the treatment groups.

Data from the Focus Forming Unit (FFU) Assay and the Microtiter Tetrazolium (MTT) Assay were evaluated with univariate analysis. The results are presented as percentages to reflect the levels of viral inhibition and cell viability, respectively.

Ethical clearance

Following a sequence of steps to secure ethical authorization, this study was executed. The Research Ethics Committee of the Faculty of Medicine at Swadaya Gunung Jati University (UGJ) issued the ethical approval, referenced as 11/EC/FKUGJ/IV/2024.

3. Results

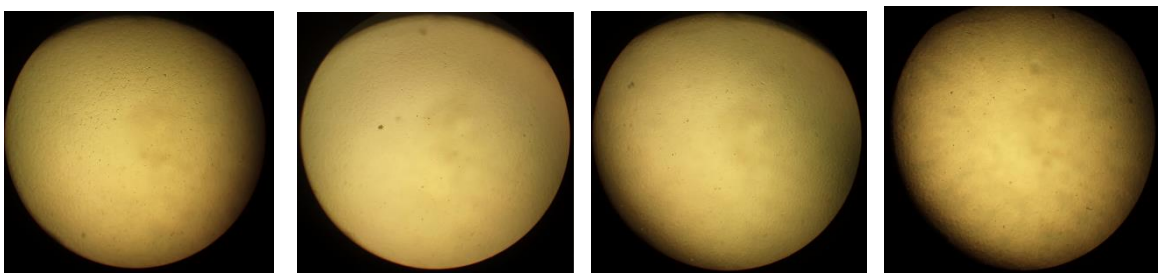
Cell viability

Table 1 displays the calculated percentages of cell viability for both the treated groups and the control group. As presented in Table 1, nicotiflorin derived from cassava leaves (**Manihot esculenta** Crantz) exhibited the lowest cytotoxic effects on Vero cells at concentrations of 10 µg/mL and 5 µg/mL, with cytotoxicity values of 47.77% and 34.63%, respectively. Lower cytotoxicity values indicate a higher percentage of cell viability, suggesting that nicotiflorin is less harmful to Vero cells at these concentrations.

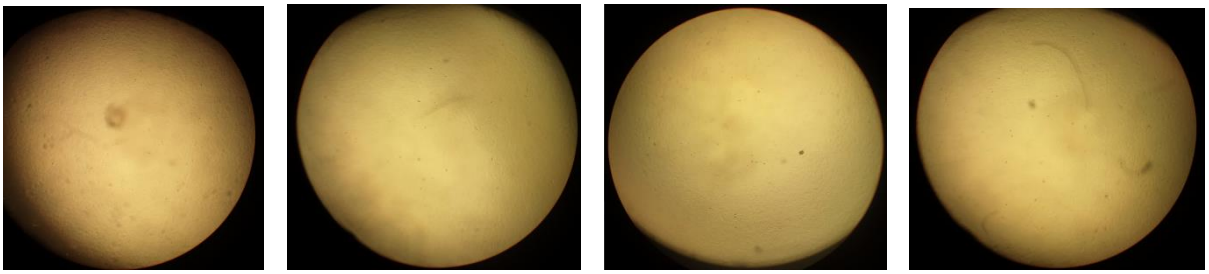
Table 1. Percentages of cell viability after treated with various concentrations of nicotiflorin

NO	Conc. (µg/ml)	Nicotiflorin			Cell Viability (%)	Cytotoxicity (%)
		P1	P2	P3		
1	80	0.327	0.319	0.330	32.55	67.45
2	40	0.355	0.341	0.368	35.46	64.54
3	20	0.352	0.399	0.383	37.80	62.20
4	10	0.536	0.498	0.533	52.23	47.77
5	5	0.709	0.674	0.578	65.37	34.63
6	2.5	0.285	0.299	0.299	29.44	70.56

In Figure 1, this is the result of the MTT assay test, where it can be observed that the higher the concentration of the compound, the paler the color appears. A light yellow or pale color, as seen in the image, indicates low cell viability or that many cells have died. In contrast, a dark purple color indicates high cell viability, meaning that many cells are alive and capable of forming formazan. Moreover, Figure 2 shows that the CC50 value for nicotiflorin derived from cassava leaves (*Manihot esculenta* Crantz) is 19.24 µg/mL, suggesting that nicotiflorin is non-toxic to Vero cells at concentrations under 19.24 µg/mL.



Nicotiflorin 80 µg/mL Nicotiflorin 40 µg/mL Nicotiflorin 20 µg/mL Nicotiflorin 10 µg/mL



Nicotiflorin 5 µg/mL Nicotiflorin 2.5 µg/mL Nicotiflorin 1.25 µg/mL DMSO 100µL

Figure 1. *Microculture Tetrazolium Technique* (MTT Assay) results

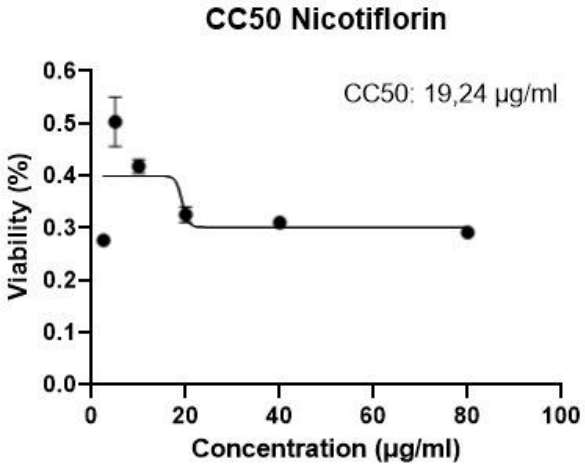


Figure 2. Nonlinear regression curve of cell viability

Inhibitory activity against DENV-1

The FFU assay results were showed in Table 2. The recent results reveal that nicotiflorin, a flavonoid from cassava leaves (*Manihot esculenta* Crantz), efficiently suppresses DENV-1 replication across concentrations between 1.5 µg/mL and 50 µg/mL.

Table 2. Percentages of denv-1- infected cell after treated with various concentrations of nicotiflorin

Concentration (µg/mL)	Inhibition per well (%)			Infectivity (%)
50	100	97.5	97.5	2.0
25	87.5	90	90	0.7
12.5	80	90	87.5	4.0
6.25	75	9.5	82.5	2.7
3.125	90	92.5	95	4.0
1.5	62.5	30	57.5	7.3

From Figure 3, the image shows a positive result of the FFU assay, with a considerable number of infection foci, indicating the presence of an active virus in the sample. The FFU assay data is later used to calculate the viral titer in FFU/mL, using a formula based on the number of foci, inoculum volume, and dilution factor. The more foci/spots that are formed, the higher the viral titer in the test sample.

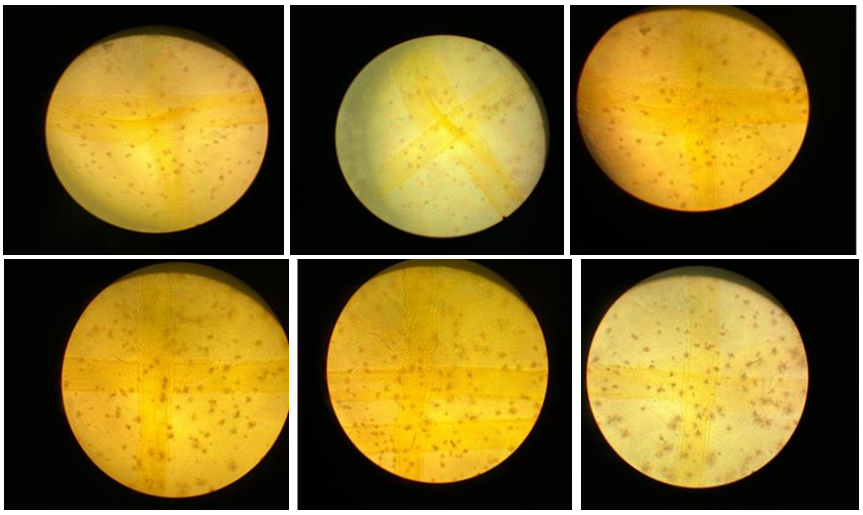


Figure 3. *Focus Forming Unit Assay* (FFU) results

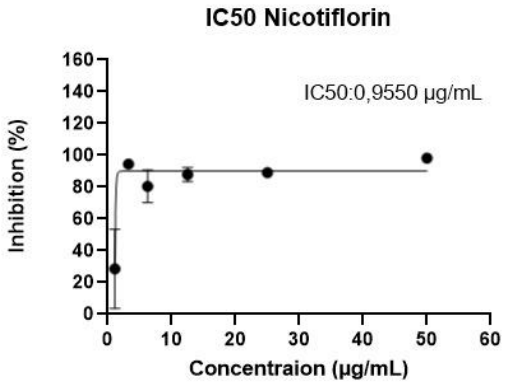


Figure 4. Nonlinear regression curve of DENV-1 replication inhibiton by nicotiflorin of cassava leaf

Selectivity index

Table 3 shows that the selectivity index (SI) was calculated by dividing the CC_{50} by the IC_{50}, resulting in an SI value of 20.14. This means that the nicotiflorin concentration from cassava leaf (*Manihot esculenta* Crantz) causing 50% cytotoxicity is 20.14 times greater than the concentration required to achieve 50% inhibition of DENV-1 antiviral activity. At this level, nicotiflorin suppresses DENV-1 infectivity while preserving cell viability.

Table 3. Selectivity Index Calculation

Selectivity Index		
$SI = \frac{\text{CC50}}{\text{IC50}}$	$SI = \frac{19.24}{0.9550}$	$= 20.14$

Statistical analysis result

Table 4 illustrates that the normality assessment via the Shapiro-Wilk test yielded a P-value of 0.000 (P<0.05), indicating non-normal data distribution. The hypothesis was tested using the Kruskal-Wallis method, which produced a P-value of 0.002 (P<0.05), thereby confirming the study's hypothesis. Statistical differences between the treatment groups were identified through Post Hoc analysis (P > 0.05), whereas a notable distinction was found between the treatment groups and the 0.2% DMSO negative control, as evidenced by a P-value of 0.000 (P < 0.05).

Table 4. Results of statistical analysis

	Sig.
Shapiro Wilk test	0.000
Kruskal Wallis test	0.002

4. Discussion

An optimal antiviral compound for managing DENV infection must display low toxicity to the host cells and possess strong potency in suppressing viral replication. This type of antiviral would efficiently diminish the virus's ability to replicate inside host cells while maintaining the health and survival of those cells. (Lal S et al., 2021)

According to this study, the inhibitory concentration test of nicotiflorin flavonoid from cassava leaves (*Manihot esculenta* Crantz) demonstrated its effectiveness in inhibiting dengue virus replication. For effective treatment of DENV infection, the ideal antiviral compound must show low harmful effects on host cells while possessing strong effectiveness in blocking viral replication. This antiviral agent would reduce the virus's replication inside host cells without negatively impacting the survival of those cells (Badshah et al., 2021). According to the outcomes of the inhibitory concentration assay of the flavonoid nicotiflorin as shown in Table 2, all tested concentrations exhibited an effect in inhibiting dengue virus replication. According to Table 2, the highest percentage of inhibition was observed at a concentration of 50 µg/mL with an an inhibition rate of 97.5%. Meanwhile, the lowest inhibition rate was observed at a concentration of 1.5 µg/mL with an infectivity value of 57.5%. These findings indicate that the inhibitory activity of nicotiflorin flavonoid extracted from cassava leaves (*Manihot esculenta Crantz*) against DENV-1 is considered high.

As displayed in Table 2, a non-linear suppression of DENV-1 replication was perceived at a specific concentration of 3.125 µg/mL of nicotiflorin derived from cassava leaves (*Manihot esculenta* Crantz), showing an infectivity rate of 4.0%. In theory, increased levels of a compound typically result in stronger suppression effects, whereas reduced levels tend to cause milder responses (Guérard et al., 2015). The ability of nicotiflorin to suppress DENV-1 replication can be affected by various factors. The observed non-linear dose-response relationship may be attributed to the presence of poorly soluble particles, which can cause DNA damage through various complex mechanisms such as antioxidant capacity, DNA repair ability, and selective proliferation and apoptosis. The complexity of these variables makes it challenging to determine the exact mechanisms, thus requiring further investigation.

In this study, the cytotoxicity and antiviral activity of nicotiflorin from cassava leaves (*Manihot esculenta* Crantz) against Vero cells and DENV-1 replication were determined based on CC_{50} and IC_{50} values. The results showed that the CC_{50} was 19.24 µg/mL and the IC_{50} was 0.9550 µg/mL. A study conducted by (Rayasari et al., 2024) utilizing quercetin, another flavonoid isolated from cassava leaves, reported a CC_{50} of 3.044 µg/mL and an IC_{50} of 0.025 µg/mL against DENV-1. The differences in CC_{50} values between the present study may be due to the different types of flavonoids used. This study employed pure nicotiflorin, whereas Rayasari et al. (2024) used pure quercetin, both derived from cassava leaves and tested on Vero cells.

Compared to quercetin, nicotiflorin from cassava leaves exhibited a higher CC_{50} value, indicating lower cytotoxicity. This may be related to differences in the chemical structure of the compounds. Pure, unmodified compounds may interact more strongly with cellular components, thereby increasing their cytotoxicity (Bolívar-Marin et al., 2022). In this study, the predicted IC_{50} value of nicotiflorin was 0.9550 µg/mL. In comparison to quercetin with an IC_{50} of 0.025 mg/dL, nicotiflorin from cassava leaves demonstrated a higher IC_{50}, indicating lower effectiveness in inhibiting DENV-1 replication. A higher IC_{50} value means a greater concentration is required to inhibit 50% of viral replication compared to quercetin.

This comparison indicates that nicotiflorin from cassava leaves (*Manihot esculenta* Crantz) has a higher CC_{50} value (19.24 µg/mL) than quercetin (3.044 µg/mL), implying lower toxicity towards Vero cells. However, the IC_{50} value of nicotiflorin (0.9550 µg/mL) is also higher than that of quercetin (0.025 µg/mL), suggesting that nicotiflorin is less effective in inhibiting DENV-1 replication. Thus, while nicotiflorin appears safer for host cells, its antiviral potency is not as strong as that of quercetin.

A study done by (Dewi et al., 2020) using synthetic quercetin as an antiviral against DENV-2 reported a CC_{50} value of 217.113 µg/mL and an IC_{50} value of 18.406 µg/mL. These results show that nicotiflorin exhibits higher toxicity (lower CC_{50}) but greater antiviral efficacy (lower IC_{50}) compared to synthetic quercetin. In other words, nicotiflorin can inhibit DENV-1 replication at lower concentrations. Although the IC_{50} value of nicotiflorin from cassava leaves (*Manihot esculenta* C.) is relatively higher than that of quercetin, indicating lower antiviral potency, it still demonstrates moderate toxicity based on its CC_{50} value of 19.24 µg/mL.

The analysis of the selectivity index (SI), calculated as the ratio of CC_{50} to IC_{50}, revealed that nicotiflorin has a favorable SI value of 20,14. Although this value is less than quercetin's (SI = 123), it is markedly greater than that of synthetic quercetin (SI = 11.8). A compound is regarded as having strong selectivity if its SI exceeds 3, whereas an SI of 3 or below denotes weak selectivity (Lica et al., 2021).

Limitation

This study has several limitations. Firstly, it did not assess the effects of nicotiflorin flavonoid extracted from cassava leaves (*Manihot esculenta* Crantz) at concentrations below 1.5 µg/mL. As a result, the IC50 value reported remains an estimate, and its accuracy in representing the concentration required to inhibit 50% of DENV-1 replication cannot be definitively confirmed. Secondly, the study did not explore the specific mechanism by which nicotiflorin inhibits the virus, leaving it uncertain whether the compound acts during the viral entry phase or the release phase from host cells. Lastly, because the flavonoid compounds were obtained from commercial sources, the exact origin of the cassava plants used is not identified.

5. Conclusion

This study's findings reveal that the half-maximal cytotoxic concentration (CC_{50}) of nicotiflorin derived from cassava leaves (*Manihot esculenta* Crantz) is 19.24 µg/mL, while its half-maximal inhibitory concentration (IC_{50}) against DENV-1 is around 0.9550 µg/mL. This yields a selectivity index (SI) of 20.14, indicating that nicotiflorin possesses promising potential as an inhibitory agent against DENV-1 replication. However, further research is required to elucidate the underlying mechanism by which nicotiflorin inhibits DENV-1 replication and to establish the optimal dosage that effectively suppresses viral activity without inducing cytotoxic effects. In addition, in vivo studies are necessary to evaluate the antiviral efficacy and safety of nicotiflorin when administered as a therapeutic agent against DENV-1 in living organisms.

Conflict of Interest

No competing interests exist, no information requires declaration.

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