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The Effectiveness of Cassava Leaf (*Manihot esculenta* C.) Flavonoid Quercetin as Dengue Virus-1 Antivirus in Vitro

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ABSTRACT

Background: Dengue fever is a mosquito-borne disease that can cause severe symptoms and death, placing high burden on healthcare systems in tropical regions. Currently, there are no antiviral treatments. It has been proven that synthetic quercetin able to improve body performance and reduce the risk of infection as well as inhibit DENV replication. Cassava leaves (*Manihot esculenta* C.) contain anthocyanins (flavonoids) such as quercetin and it shows potential as antiviral agent as the synthetic as well.

Aims: To describe the levels of self-compassion and procrastination among medical students and to examine how these factors relate to academic performance.

Methods: This experimental study used post-test only with control group design consisted of 13 treatment groups and 1 control group. Treatment group were divided into 6 different concentrations for inhibition test (50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5 µg/mL) and 7 different concentrations for cytotoxicity test (80 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL). The control group was a negative control treated with 0.2% DMSO. Inhibition was assessed by Focus Forming Unit Assay and cytotoxic was assessed by number of Microtiter Tetrazolium Assay (MTT Assay). The statistical analysis used in this study were the Shapiro-Wilk test for normality test, Kruskal-Wallis test for hypothesis test, and post-hoc test to determine whether the groups studied in this research had significant differences from each other.

Results: The CC50 and IC50 values of quercetin of cassava leaf (*Manihot esculenta* C.) were found to be 3.44 µg/mL and 0.25 µg/mL, respectively, with an SI value of 123. Based on these results, it can be concluded that the flavonoid quercetin of cassava leaf (*Manihot esculenta* C.) exhibits selective properties in inhibiting the replication of DENV-1. Statistical analysis showed non-normal distribution ($P<0.05$), hypothesis test was accepted ($P<0.05$), and no significant differences between concentrations in the post-hoc test.

Conclusion: Quercetin of cassava leaf (*Manihot esculenta* C.) is effective as an antiviral agent against dengue serotype 1 strain New Guinea C in vitro.

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

Dengue fever is an acute febrile illness caused by the bite of an *Aedes aegypti* mosquito infected with one of four dengue virus serotypes: dengue virus serotype 1 (DENV-1), dengue virus serotype 2 (DENV-2), dengue virus serotype 3 (DENV-3), and dengue virus serotype 4 (DENV-4) (Wang W et al., 2020). Dengue virus infection can lead to plasma leakage and death. According to the WHO in 2019, DHF is a tropical disease that has long been an international health problem, and the worldwide incidence of DHF has continued to increase 30-fold over the past 50 years. (Wong P et al., 2019; Roy SK et al., 2021)

Dengue remains a major public health problem in Indonesia. By the end of 2022, Indonesia reprted 143.000 dengue cases since data collection began in 2006, with the highest number of dengue cases in the provinces of West Java, East Java and Central Java (Saputra, 2021; Rathnayake *et al*., 2019)

Currently, treatment of dengue infection is still supportive, such as fluid replacement to prevent dehydration and antipyretics to reduce fever. Unfortunately, due to the various complications that can occur, the use of dengue vaccine as an antiviral is still very limited. (B E Dewi *et al*., 2020)

Herbal plants have been used as medicines since ancient times. One of them is the cassava plant (*Manihot esculenta* C.) which often found in the Asian continent, including Indonesia. (Tsige et al., 2019; Fachriyah E *et al*., 2023) Some studies show that cassava leaves (*Manihot esculenta* C.) contain several antioxidant compounds such as β-carotene (23 - 86 mg/100 g), vitamin C (1.7 - 419 mg/100 g), vitamin A, anthocyanins (flavonoids), saponins, steroids and glycosides. In Nigeria, the cassava leaves (*Manihot esculenta* C.) are used to treat skin diseases, tumors, abscess conjunctivitis, rheumatic fever, headache, diarrhea, and loss of appetite. (Widiastuti D, 2019) While in Indonesia various studies have examined the bioactivity of cassava leaf. Research by Rikomah et al. found that a 70% ethanol extract of cassava leaf demonstrated an antirheumatic effect in mise (*Mus musculus*), witj the optimal dose being 0.65 mg. The ethanol extract also showed diverse pharmacological activites, including antibacterial, analgesic antidiarrheal, blood-sugar lowering, anthelmintic, and antioxidant effects. (Mustarichie R et al., 2020; Pratiwi AP, 2016; Azmi P *et al*., 2022)

One of the many pure compounds found in nature is quercetin. Quercetin has long been used by several researchers as an anti-inflammatory, antibacterial, antiviral, and antifungal agent.m A study by Wang et al. (2017) reported that the flavonoid content of quercetin found in cassava leaf (*Manihot esculenta* C.) ranged from 0.7 mg/L to 93.8 mg/L. Quercetin is known to be a potent anticancer agent in humans; recently, quercetin was found to inhibit the growth of leukemia cells. In addition, flavonoids have been reported to have antiviral activity against adenovirus, HSV-1 (herpes simplex virus-1), HSV-2 (herpes simplex virus-2), and HCMV (human cytomegalovirus), as well as hepatitis C virus. These viruses share the ability to manipulate host immune responses to persist within the host and maintain latent infections, while hepatitis C virus and dengue virus are both RNA viruses. (Wang Dm et at., 2017; Thagriki D S; 2022)

Dengue virus serotype 1 (DENV-1) can growth rapidly in cell cultures. The virus's envelope and capsid proteins, ensures consistent replication, providing a reliable framework for testing potential therapeutic agents, which can also inform strategies against other DENV serotypes and similar viruses. Research on quercetin of cassava leaf (*Manihot esculenta* C.) as antiviral has not been widely reported in Indonesia. Therefore the effectiveness flavonoid quercetin of cassava leaf (*Manihot esculenta* C.) as DENV-1 antiviral and its cytotoxicity on Vero cells was carried out in this study (Coffey et al., 2015).

2. Methods

Study design/ Research procedures

This study was granted with an ethical approval by the Ethics Committee for Research of the Faculty of Medicine of the Swadaya Gunung Jati University with the letter number 16/EC/FKUGJ/IV/2024. This research is an experimental research with a "posttest only with control group design" using dengue virus serotype 1 and vero cell as the object of research. This research consisted of 13 treatment groups and 1 control group. The treatment group was divided into 6 different concentrations of quercetin of cassava leaf (*Manihot esculenta* C.)

for inhibition test and 7 different concentrations of quercetin of cassava leaf (*Manihot esculenta* C.) for cytotoxicity test which carried out using simple random sampling method. This research was conducted at the Clinical Microbiology Laboratory, Department of Microbiology, Faculty of Medicine, Universitas Indonesia in Cikini, Central Jakarta, from May 2024 to June 2024. The number of repetitions was determined using Federer's formula, with value of r (the number of repetitions) was to be 3.5. The inclusion criteria of this research are dengue virus serotype 1 strain New Guinea C grown on cell growth medium and dengue virus with multiple of infection (MOI) 0.2. While the exclusion criteria in this study is cell growth medium contaminated with various microorganisms other than dengue virus.

Measurements

a. Materials

In this research, the flavonoid compound quercetin was obtained commercially from MarkHerb Manufacturer, with catalog number FLV-1-100. The dengue virus and vero cells used in this research are a collection from Microbiology laboratory, Faculty of Medicine, Universitas Indonesia with the passage number of 4 and 2 respectively.

b. Determination of cytotoxicity through CC_{50} value

MTT assay method was used to evaluate the cytotoxicity of quercetin flavonoids on Vero cells after treated with different concentrations of quercetin by the percentage of cell viability. Cell viability defined as the ability of cells to survive. In this study cell viability was assessed using the MTT assay. This method quantifies cell viability by measuring optical density or absorbance. Higher absorbance values observed in wells with more intense coloration indicates greater cell viability. Cell absorbances were determined by an ELISA reading in which the absorbance value determines the value of the cell viability with 450 nm wavelength.

Cells were plated in 96-well plates and treated with quercetin extracted from cassava leaves at concentrations of 80 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2,5 µg/mL, and 1,25 µg/mL. After treatment, the cells were incubated at 37°C for 48 hours. The medium was then replaced with MTT solution and the cells were incubated for an additional 2 hours. MTT results are processed using Graphpad Prism software to calculate the half-toxicity concentration (CC_{50}) value.

c. Determination of antiviral activity through IC_{50} value

The inhibition of denv-1 replication by flavonoid quercetin of cassava leaf (*Manihot esculenta* C.) was assessed by the percentage of infectivity using the FFU method. Cells infected with denv-1 appear as brown foci. In this procedure, various concentrations of quercetin were administered to Vero cells infected with dengue virus. FFU results were processed using Graphpad Prism software to calculate the half-inhibitory concentration (IC_{50}) value.

Cells were treated with quercetin of cassava leaf at concentrations of 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, and 1.5 µg/mL while exposed to viruses at a multiplicity of infection (MOI) of 0.2. After a two-hour incubation at 37°C, the viruses were removed by washing the cells with 200 µL of DMEM. Cells were then incubated for 72 hours at 37°C and the supernatants were collected for virus titration.

d. Selectivity index

Selectivity index of quercetin was calculated using the formula CC_{50}/IC_{50}.

Statistical techniques

Data obtained from this research were statistically analyzed using computer software. Normality tests were performed using the Shapiro-Wilk test because the sample size was less than 50. Hypothesis testing was performed using the nonparametric Kruskal-Wallis test. A post hoc test was then performed to determine whether the groups studied in this research had significant differences from each other. Focus Forming Unit Assay (FFU Assay) and Microtiter Tetrazolium Assay (MTT Assay) data were analyzed using univariate analysis. Data are expressed as percentages.

Ethical clearance

This study was conducted after undergoing a series of procedures to obtain ethical approval. Ethical clearance was issued by the Research Ethics Committee of the Faculty of Medicine, UGJ, with reference number 16/EC/FKUGJ/IV/2024.

3. Results

Cell viability

Cell viability percentages of the treated groups and control group were counted in Table 1.

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

Concentration (μg/mL)	Cell Viability (%)			Cytotoxicity (%)
80	0.459	0.507	0.371	43.44
40	0.391	0.476	0.346	49.87
20	0.393	0.446	0.392	48.94
10	0.366	0.422	0.319	55.37
5	0.553	0.400	0.442	40.44
2.5	0.685	0.462	0.519	26.39
1.25	0.732	0.575	0.588	14.52
DMSO 100μL	0.685	0.772	0.718	0.000

As shown in Table 1, quercetin of cassava leaf (*Manihot esculenta* C.) has the lowest level of cytotoxicity towards Vero cells at concentrations of 2.5 μg/mL and 1.25 μg/mL with cytotoxicity values of 26.39% and 14.52%. The lower cytotoxicity values of the flavonoid quercetin towards Vero cells, the higher percentage of cell viability. From Figure 1, MTT assay results was detected using a spectrophotometer at a wavelength 450 nm, this technique measures optical density or absorbance. Higher absorbance values corresponded to wells with more intense coloration, indicating a greater number of viable cells.

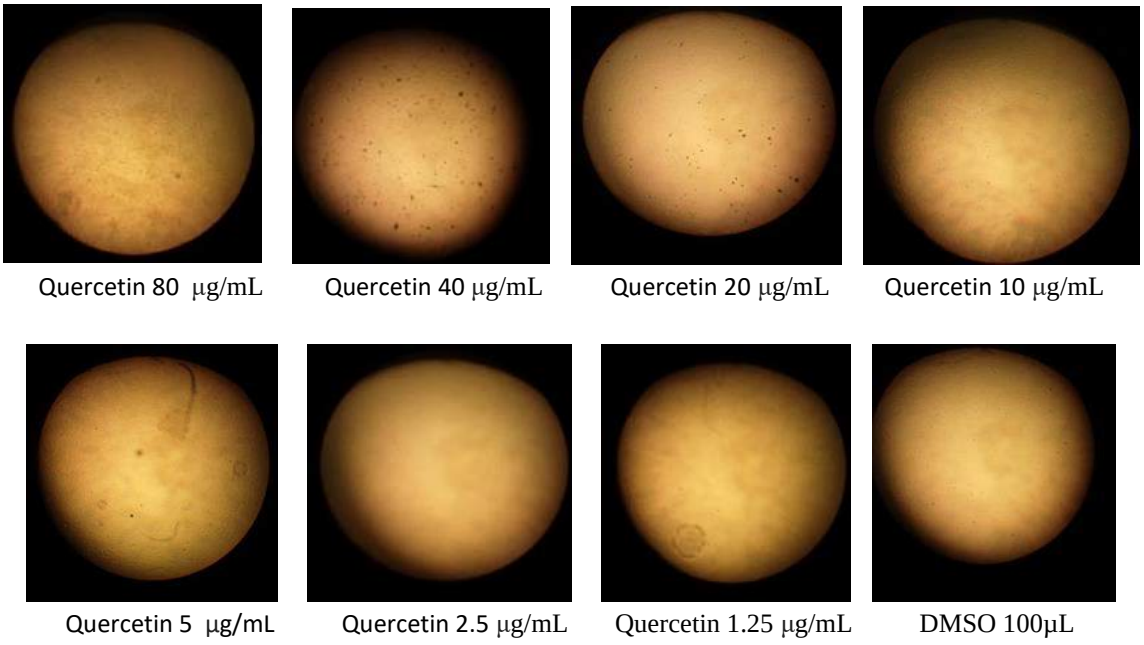

Figure 1. MTT Assay Results

From Figure 2, the CC_{50} value of quercetin of cassava leaf (*Manihot esculenta* C.) was 3.044 µg/mL indicating that quercetin of cassava leaves is non-toxic to Vero cells at concentrations below 3.44 µg/mL.

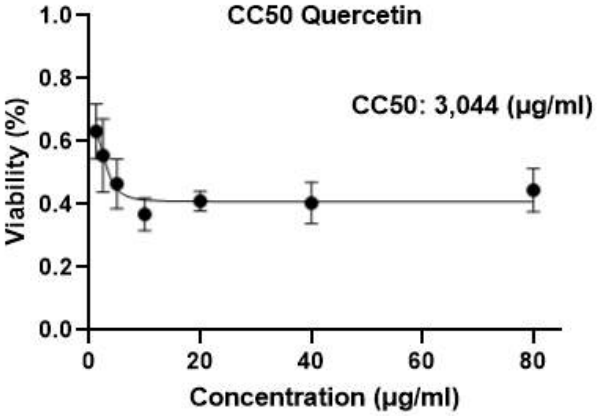

Figure 2. Nonlinear regression curve of cell viability

Effectivity of DENV-1 inhibition

The FFU assay results were showed in Table 2. it can be observed that flavonoid quercetin of cassava leaf (*Manihot esculenta* C.) at concentrations ranging from 50 µg/mL to 1.5 µg/mL effectively inhibits DENV-1 replication with infectivity levels reduced to ≤3.5%. The highest inhibitory activity of quercetin of cassava leaf (*Manihot esculenta* C.) against DENV-1 replication was observed at concentrations of 50 µg/mL and 25 µg/mL with infecticity values of 0.922% and 0.264%, respectively. In contrast the lowest inhibitory activity was found at concentration of 1.25 µg/mL with an infectivity valaue of 90.91%.

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

Concentration (µg/mL)	Inhibition per well (%)			Infectivity (%)
50	100	95	99	0.922
25	99	100	99	0.264
12,5	96	95	97	3.953
6,25	98	97	97	2.889
3,125	96	95	97	4.216
1,5	92	90	96	9.091

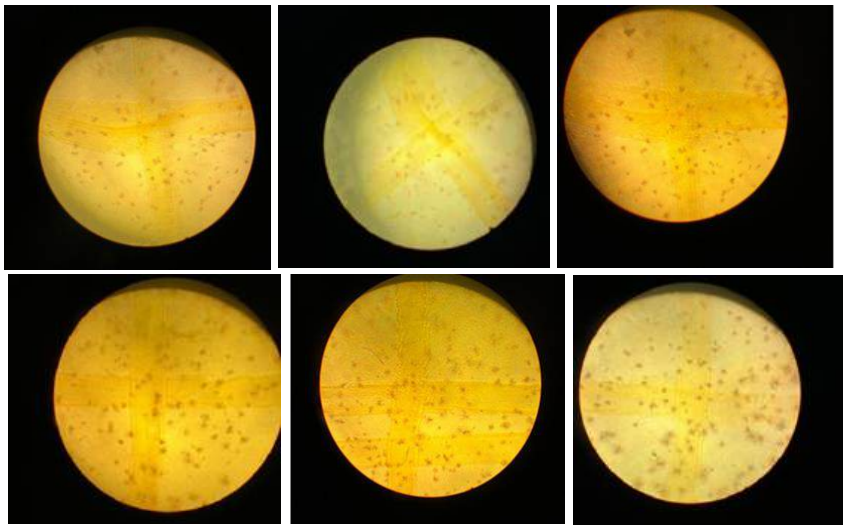

Figure 3. FFU results

Based on Figure 3, it can be concluded that cells infected by denv-1 will absorb the brown color and will be seen as brown foci. The more brown foci that are visible, it indicates that more cells are infected.

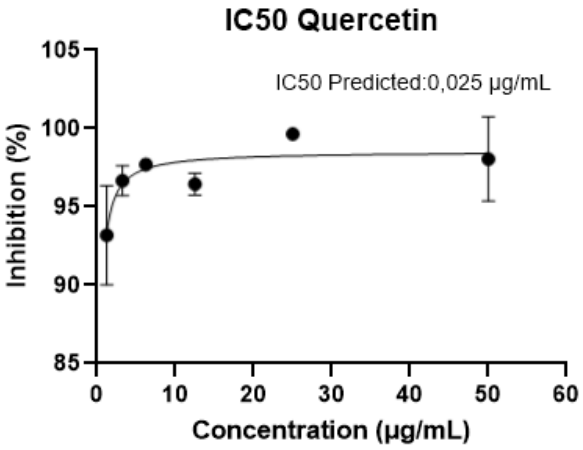

Figure 4. Nonlinear regression curve of DENV-1 replication inhibiton by quercetin of cassava leaf

Selectivity index

The selectivity index (SI) in this study was determined by dividing the CC_{50} value by the IC_{50} value, and found that SI value was 123. This indicates that the concentration of quercetin of cassava leaf (*Manihot esculenta* C.) required to achieve 50% cytotoxicity is 123 times higher than the concentration needed to cause 50% antiviral activity against DENV-1. At this concentration, quercetin of cassava leaf (*Manihot esculenta* C.) effectively inhibits DENV-1 infectivity while maintaining cell viability.

Statistical analysis result

In the normality test results using Shapiro Wilk test, a P-value of 0.000 ($P<0.05$) indicated that the data was not normally distributed. The hypothesis test using Kruskal Wallis test, resulted P-value of 0.002 ($P<0.05$), supporting the research's hypothesis. Post Hoc analysis showed significant differences between treatment groups ($P>0.05$) expect for the 0.2% DMSO negative control group, which had a significant difference compared to the treatment groups with a P-value of 0.000 ($P<0.05$).

4. Discussion

The ideal antiviral agent for treating DENV infection should exhibit minimal cytotoxicity towards host cells while demonstrating high efficacy in inhibiting viral replication. Such an antiviral would effectively reduce viral replication within host cells while preserving cell viability (Lal S et al., 2021). Several factors may influence the inhibitory ability of quercetin against DENV-1 replication. Based on the results presented in Table 2, there is one concentration of quercetin from cassava leaves (*Manihot esculenta* C.) at which the inhibitory effect on DENV-1 replication is non-linear, specifically at a concentration of 6.25 µg/mL with an infectivity value of 2.899%. According to theory, higher concentrations of a compound typically result in greater inhibitory effects, while lower concentrations yield weaker inhibitory effects (M Guerard et al., 2014)

Factors that may contribute to this non-linear inhibitory outcome include the presence of pro-oxidant compounds, which can induce DNA damage due to reactive oxygen species (ROS) produced endogenously in all cell types. The second factor is the presence of poorly soluble particles, which may cause DNA damage. The third factor involves exposure to nanoparticles or nanomaterials. Lastly, nucleoside analogs may also contribute to DNA strand damage (M Guerard et al., 2014).

In this study the value of cell viability of quercetin of cassava leaf (*Manihot esculenta* C.) on Vero cells and inhibitory rates of quercetin of cassava leaf (*Manihot esculenta* C.) against DENV-1 were found through CC50 and IC50 values, the CC50 and IC50 values were found to be 3.044 µg/mL and 0.025 µg/mL respectively. Study conducted by Dewi BE et al. used querctein synthetic as antivirus against DENV-2 has shown that CC50 value for quercetin synthetic on Vero cells was to be 217.113 µg/mL and IC50 value of quercertin against DENV-1 was to be 18.406 µg/mL. The variation in CC50 values between this study and study conducted by Dewi BE et al., it may be attributed to differences in the cell lines and quercetin types used. This study employed Vero cells and utilized pure compound quercetin of cassava leaf (*Manihot esculenta* C.), while Dewi BE et al. used Huh 7it-1 human cell line and synthetic quercetin. Compared to synthetic quercetin, quercetin of cassava leaf (*Manihot esculenta* C.) demonstrated a lower CC50 value, it's indicating a higher level of toxicity, it may be due to the sensitivity of vero cells used in this research. Furthermore, chemical structure may lead to higher pure compound's cytotoxicity value. The chemical structure of pure compound is unmodified which may lead to stronger interactions with cellular components (Bol S et al., 2022). In this study, the predicted IC50 value was 0.025 µg/mL. When compared to synthetic quercetin, quercetin derived of cassava leaf (*Manihot esculenta* C.) exhibited a lower IC50 value which has 3.044 mg/dL as IC50 value, suggesting that quercetin of cassava leaf (*Manihot esculenta* C.) is more effective in inhibiting DENV-1 replication by 50% in lower dose.

A study by Maharani et al. (2020) investigated the antiviral effects of morin against DENV-1. Morin (2-(2.4-dihydroxyphenyl)-3.5.7-trihydroxychromen-4-one) is a flavones in several species of the Moraceae family, In that study, one of the antiviral indicators was evaluated based on the cytotoxic activity of morin on Vero cells, represented by the CC50 value and the inhibitory activity of DENV-1 infection by quercetin was evaluated using IC50 value. The results showed that CC50 value of morin on Vero cells was 12.46 µg/mL and IC50 value of morin against DENV-1 was 9.42 µg/mL (Maharani A et al., 2020). When comparing CC50 and IC50 values of cassava leaf quercetin (*Manihot esculenta* C.), the cytotoxic activity of cassava leaf quercetin showed a lower CC50 value of 3.044 mg/mL, indicating that quercetin of cassva leaf (*Manihot esculenta* C.) is more toxic to Vero cells than morin compound. However, in terms of DENV-1 infection inhibition, quercetin of cassava leaf (*Manihot esculenta* C.) demonstrated a lower IC50 value of 0.25 mg/mL compared to morin, which has an IC50 value of 9.42 mg/mL. This suggests that quercetin of cassava leaf (*Manihot esculenta* C.) can inhibit 50% DENV-1 infection at a lower dose than the morin compound.

Quercetin, a flavonoid derivate, exhibits promising antiviral properties. According to a study by Zandi et al., quercetin effectively inhibits DENV-2 replication, reducing RNA copy production by 67% at a concentration of 50 µg/mL. The study also reported the IC50 value of 35.7 µg/mL for quercetin. (Zandi K et al., 2011) A study docking conducted by Datu et al., quercetin derived from miana leaves (Coleus scutellarioides) is also considered as dengue virus NS5 protein inhibitor.

Although the CC50 value of cassava leaf quercetin (*Manihot esculenta* C.) is relatively low compared to synthetic quercetin and morin, it's indicating a higher cytotoxicity at a dose equivalent to its CC50 of 3.044 mg/dL, an analysis of the selectivity index (SI), calculated as the ratio of CC50 to IC50, reveals that cassava leaf of quercetin (*Manihot esculenta* C.) exhibits an excellent SI value of 123. This is significantly higher than the SI of synthetic quercetin, which is 11.8. A compound with an SI >3 is considered to have good selectivity, while an SI ≤3 indicates poor selectivity (Muhammad D et al., 2019)

Limitation

The limitations of this study include the lack of testing of quercetin flavonoid from cassava leaves (*Manihot esculenta* C.) at concentrations below 1.5 µg/mL, which means that the obtained IC50 is still a prediction and its validity for inhibiting DENV-1 replication by 50% cannot be confirmed. Additionally, the study did not investigate the mechanism of viral inhibition, leaving it unclear whether quercetin from cassava leaves inhibits DENV-1 replication during viral entry into cells or during viral release from cells. Furthermore, since the compounds used in this study were commercially sourced, the specific origin of the utilized cassava plants remains unknown.

5. Conclusion

The findings of this study indicate that quercetin of cassava leaf (*Manihot esculenta* C.) has a half-maximal cytotoxic concentration (CC_{50}) of 3.044 µg/mL and a half-maximal inhibitory concentration (IC_{50}) of approximately 0.025 µg/mL against DENV-1, resulting in a selectivity index (SI) of 123. This suggests that quercetin of cassava leaf (*Manihot esculenta* C.) holds significant potential as an antiviral agent against DENV-1. Further research is needed to explore the mechanism by which quercetin derived from cassava leaves inhibits DENV-1 replication and to determine the effective dosage that can inhibit DENV-1 replication without being toxic to cells. Additionally, future studies should be conducted in vivo to evaluate how quercetin functions as an antiviral compound against DENV-1 when administered as an antiviral agent in vivo.

Conflict of Interest

There is no conflict of interest-nothing to disclosure.

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