

In Vitro Antiviral Activity of Dried Red Jujube Fruit (*Ziziphus jujuba*) Ethanol Extract against DENV-2

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ABSTRACT

Dengue infection has become one of the most important mosquito-borne diseases worldwide and is caused by the dengue virus (DENV). Recently, neither patent drug, phytopharmaceutical medicine, nor standardized herbal medicine has been officially available against DENV. Dried red jujube fruit (*Ziziphus jujuba*) ethanol extract has been proven to have an antiviral effect, anti-inflammatory efficacy, and antioxidant properties, which have potential activity against DENV infection. This research was conducted to analyze the antiviral activity of dried red jujube fruit ethanol extract against DENV-2 in vitro. The half-maximal cytotoxic concentration (CC₅₀) and half-maximal inhibitory concentration (IC₅₀) were examined on Vero cells by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, whereas the half-maximal effective concentration (EC₅₀) was determined using luminescence assay. The selectivity index (SI) value was determined from the ratio of CC₅₀ and EC₅₀. Dried red jujube fruit ethanol extracts inhibited DENV-2 in 24.59%, 22.39%, 17.98%, 14.5%, 6.42%, and 1.28% at 80 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, and 2.5 µg/mL, respectively. The extract exhibited antiviral activity against DENV-2, showing a CC₅₀ of 67.73 µg/mL, an IC₅₀ value of 166.18 µg/mL, and an EC₅₀ of 64.87 µg/mL, with an SI of 1.04. The LD₅₀ value was 707.95 mg/kg. Dried red jujube fruit ethanol extract could be a potential candidate for developing an antiviral against DENV-2.

Keywords: *Ziziphus jujuba*, dried red jujube fruit, dengue virus, DENV-2, antiviral.

INTRODUCTION

Dengue is a primary arboviral global concern infection in tropical and subtropical regions, transmitted by *Aedes albopictus* and *Aedes aegypti* as their vectors. Humans can be infected through the bite of infected female mosquitoes. Dengue virus (DENV) consists of several serotypes, namely DENV-1, DENV-2, DENV-3, DENV-4, and more recently DENV-5.¹ Each DENV serotype can cause an acute febrile illness with several different clinical symptoms, ranging from dengue fever (DF) to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).²

Dengue infection has become one of the most important mosquito-borne diseases worldwide. The incidence of dengue infection, according to reported cases to the World Health Organization (WHO), increased from around 500,000 cases in 2000 to 5.2 million cases in 2019.³ The disease is endemic in more than 100 countries, with Asia presenting approximately 70% of the global disease burden. There were 390 million DENV infections per year, resulting in up to 36,000 deaths.⁴ There were at least 23 countries that reported dengue outbreaks in 2003, with approximately more than 5 million cases in 80 countries.⁵ This self-limiting disease usually only requires supportive therapy as primary therapy, but improper treatment due to inadequate sources of knowledge, the economy, or health access could worsen the disease.

Recently, neither patent drugs, phytopharmaceutical medicine, nor standardized herbal medicine have been officially available against DENV. Dried red jujube fruit (DRJF), or Chinese jujube (*Ziziphus jujuba*), has several

secondary active metabolites, such as flavonoids, phenolics, triterpenoids, and carotenoids.^{6,7} DRJF has proven to have an antiviral effect against the influenza virus, an anti-inflammatory effect, and a potential source of antioxidant properties.⁸⁻¹⁰ This research was conducted to establish the potential of DRJF ethanol extract as an antiviral against DENV-2 *in vitro*.

RESULTS AND DISCUSSION

Jujube, also known as Chinese jujube, is a fruit rich in nutrients and bioactive compounds. This fruit contains carbohydrates, proteins, fats, vitamins (A, B1, B2, B3, B6, and C), minerals (potassium, phosphorus, calcium, magnesium, sodium, iron, manganese, copper, and zinc), fiber, and bioactive compounds (phenolics, flavonoids, triterpenoids, and carotenoids).¹¹⁻¹³ Phenolics and flavonoids play the main role as antioxidants in jujube fruit.¹⁴⁻¹⁶

The maturity level of the fruit is generally divided into early, middle, and late stages. Its color can be evaluated from green at early maturity to red at late maturity. Research on fresh jujube fruits revealed that the total phenolic content (TPC) and total flavonoid content (TFC) were at their highest in early maturity and continued to decrease to the lowest in late maturity.¹⁴ The higher the level of maturity, the lower the levels of bioactive compounds.

Jujube is a fruit that is commonly found and consumed in red and dried form. The drying process affects the structure, texture, color, chemical components, and aroma of a product. Dried products also have lower nutritional value than fresh ones because several components are lost during drying. The processes that occur include degradation of proteins, Maillard

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reactions, caramelization, lipid oxidation, and degradation of water-soluble and fat-soluble vitamins. Changes in these components are influenced by temperature, method, and drying duration.¹⁷ Previous studies revealed that red jujube fruit from fresh to dried experiences decreased levels of vitamins, TPC, TFC, and antioxidant activity.^{18,19} Despite the significant component changes, DRJF has been shown to have several health benefits.^{11,20}

The highest cell viability rate was found in DRJF ethanol extract at 2.5 µg/mL, with an amount of 126.16%. The cell viability rates of DRJF ethanol extract at 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL were 104.3%, 78.81%, 72.52%, and 59.93%, respectively. The lowest cell viability rate was found in DRJF ethanol extract at 80 µg/mL, with an amount of 50.66%. Pearson correlation analysis showed a high negative correlation between the concentration of DRJF ethanol extract and the percentage of cell viability ($r = -0.809$, $p = 0.051$). The percentage of Vero cell viability decreased as the concentration of DRJF ethanol extract increased, forming a linear line, as seen in Figure 1.

The highest viral inhibition rate was found in DRJF ethanol extract at 80 µg/mL, with an amount of 24.59%. The viral inhibition rates of DRJF ethanol extract at 40 µg/mL, 20 µg/mL, 10 µg/mL, and 5 µg/mL were 22.39%, 17.98%, 14.5%, and 6.42%, respectively. The lowest viral inhibition rate was found in DRJF ethanol extract at 2.5 µg/mL, with an amount of 1.28%. Pearson correlation analysis showed a high positive correlation between the concentration of DRJF ethanol extract and the percentage of viral inhibition ($r = 0.824$, $p = 0.044$). The percentage of DENV-2 inhibition increased as the concentration of DRJF ethanol extract increased, forming a linear line, as seen in Figure 2.

The *in vitro* experimental results in Table 1 showed that DRJF ethanol extract had a CC_{50} value of 67.73 µg/mL, an IC_{50} value of 166.18 µg/mL, and an EC_{50} value of 64.87 µg/mL. The SI value from the ratio of CC_{50} and EC_{50} was 1.04. SI higher than 1 indicates that the extract efficacy against the virus is greater than the toxicity to normal cells, even though it was not a strong activity. The estimated LD_{50} value of DRJF ethanol extract was 707.95 mg/kg.

MATERIAL AND METHODS

Tools and Materials Preparation

Tools used in experimental study include biological safety cabinet (AIRTECH, Airtech Equipment Pte Ltd., Singapore), CO₂ incubator (Celculture, Esco Micro Pte. Ltd., Singapore), micropipette (Nichipet EXII Multi, Nichiryu Co., Ltd., Japan), 96-well plate (NEST,

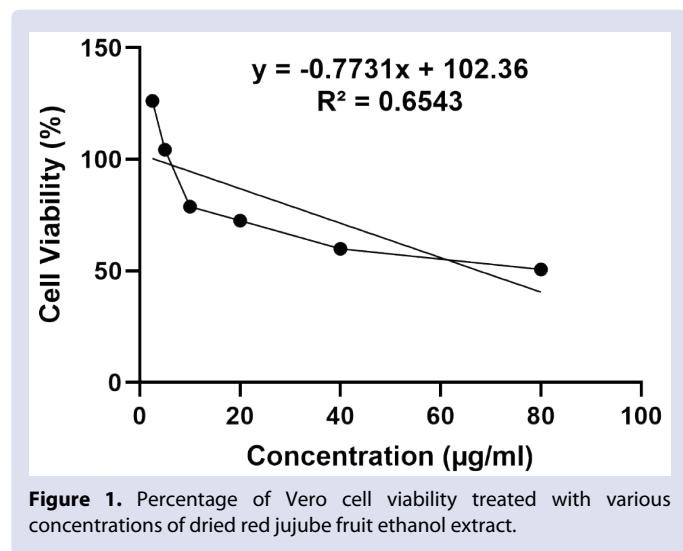


Figure 1. Percentage of Vero cell viability treated with various concentrations of dried red jujube fruit ethanol extract.

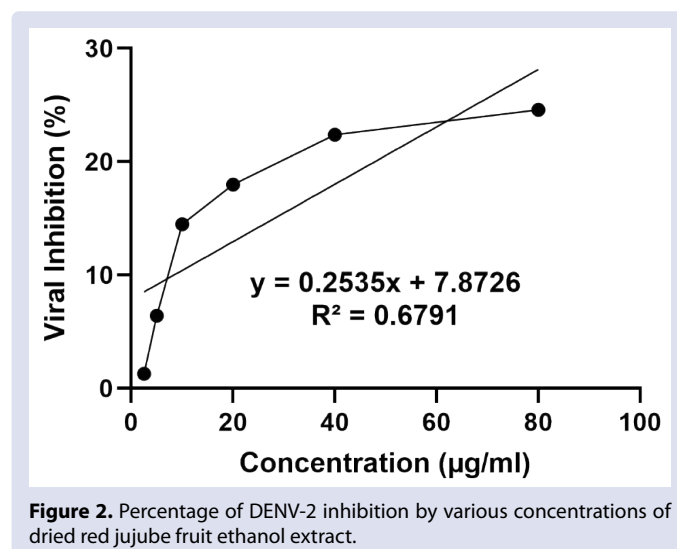


Figure 2. Percentage of DENV-2 inhibition by various concentrations of dried red jujube fruit ethanol extract.

Wuxi NEST Biotechnology Co., Ltd., China), T75 culture flask, refrigerated centrifuge, improved Neubauer counting chamber (Assistant, Hecht Glaswarenfabrik GmbH & Co. KG, Germany), light microscope (Nikon, Nikon Corp., Japan), hand tally counter, digital calculator, absorbance microplate reader (Bio-Rad, Bio-Rad Laboratories, Inc., California), luminescence microplate reader (Glomax, Promega Corp., USA), microcentrifuge tube, rotary evaporator, and oven.

Materials used in the experimental study include Vero cell, heat-inactivated DENV-3, active DENV-2, DRJF, ethanol 70%, 0.1% DMSO, and CMC-Na 1%.

Extract Preparation

DRJF were purchased from a local herbal shop and identified by the Technical Implementation Unit of the Herbal Laboratory, Materia Medica Batu, East Java, Indonesia. The DRJF ethanol extract was prepared at the Faculty of Pharmacy, Universitas Katolik Widya Mandala Surabaya, Surabaya, Indonesia, as described in the previous study.¹¹

Virus Preparation

DENV isolates were obtained from the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. The serotypes of DENV used in the experiment were active DENV-2 and heat-inactivated DENV-3. The virus titer was calculated using a hemocytometer under a light microscope and expressed in Foci-Forming-Unit (FFU). DENV-3 was inactivated using the heat treatment method at 56°C for 30 minutes.

Determination of Half-maximal Cytotoxic Concentration (CC_{50})

The half-maximal cytotoxic concentration (CC_{50}) study was performed to ensure that the DRJF ethanol extract was not toxic to the cell. The CC_{50} value was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on cell viability after being treated with the extract. 2×10^4 cells per well of Vero cells were added to a 96-well plate and incubated at 37°C with 5% CO₂. After 24 hours, Vero cells were treated with 100 µL of diluted 0.1% DMSO with six various concentrations of DRJF ethanol extract (2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL), then incubated at 37°C with CO₂ 5%. After 48 hours, 20 µL of MTT solution was added to each well and incubated for four hours. The absorbances were measured using a microplate reader at 595 nm. The percentage of cell viability was calculated as $[(A / B) \times 100]$, where A and B are the absorbance of the extract test on the cell and the absorbance of the solvent test on the

Table 1. The CC₅₀, IC₅₀, EC₅₀, Selectivity Index, and LD₅₀ values of dried red jujube fruit ethanol extract towards DENV-2 in Vero Cell.

Concentration (µg/mL)	Cell Viability (%)	CC ₅₀ (µg/mL)	Viral Inhibition (%)	IC ₅₀ (µg/mL)	EC ₅₀ (µg/mL)	Selectivity Index ^a	LD ₅₀ ^b (mg/kg)
80	50.66	67.73	24.59	166.18	64.87	1.04	707.95
40	59.93		22.39				
20	72.52		17.98				
10	78.81		14.5				
5	104.3		6.42				
2.5	126.16		1.28				

DRJF Ethanol Extract: Dried Red Jujube Fruit Ethanol Extract; CC₅₀: Half-maximal Cytotoxic Concentration; IC₅₀: Half-maximal Inhibitory Concentration; EC₅₀: Half-maximal Effective Concentration; LD₅₀: Median Lethal Dose

^a Selectivity Index = CC₅₀/EC₅₀

^b log LD₅₀ = 0.372 log IC₅₀ + 2.024

cell, respectively. The CC₅₀ value was estimated from a linear regression analysis between the percentage of cell viability and the concentration of DRJF ethanol extract.

Determination of Half-maximal Inhibitory Concentration (IC₅₀)

The half-maximal inhibitory concentration (IC₅₀) study was performed as a screening to ensure that the DRJF ethanol extract has antiviral activity against DENV-2. The IC₅₀ value was determined using an MTT assay based on viral titer after the cell was infected with DENV-2 and treated with the extract. All experiments were performed in triplicate. Vero cells were added at a density of 2 x 10⁴ cells/well in a 96-well plate and incubated at 37°C with 5% CO₂. After 24 hours, Vero cells were infected with DENV-2 at a multiplicity of infection (MOI) of 1 FFU/cell. After two hours, 100 µL of 0.1% DMSO with six various concentrations of DRJF ethanol extract were added. The plates were further incubated at 37°C with 5% CO₂ for two days, and 20 µL of MTT solution was added to each well and incubated for four hours. The absorbances were measured using a microplate reader at 595 nm. The percentage of viral inhibition was calculated as [(A - B) / (C - B) x 100%], where A, B, and C are the absorbance of the extract on the virus-infected cell, the absorbance of the virus control, and the absorbance of the cell control, respectively. The IC₅₀ value was estimated from a linear regression analysis between the percentage of viral inhibition and the concentration of DRJF ethanol extract.

Determination of Half-maximal Effective Concentration (EC₅₀)

The half-maximal effective concentration (EC₅₀) study was performed to explore the effective concentration of DRJF ethanol extract against DENV-2. The EC₅₀ value was determined by a luminescence assay using a microplate reader. The treatment was the same as previously described in the IC₅₀ study.

Determination of Median Lethal Dose (LD₅₀)

The median lethal dose (LD₅₀) value was obtained from the IC₅₀ value (µg/mL) based on a regression formula approved by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).¹² The regression formula was calculated as [log LD₅₀ = 0.372 log IC₅₀ + 2.024]. The LD₅₀ value was expressed in mg/kg.

Selectivity Index Analysis

The CC₅₀ and EC₅₀ values are the average of triplicate assays with six concentrations within the inhibitory range of the compounds. The selectivity index (SI) was determined from the ratio of CC₅₀/EC₅₀.

Statistical Analysis

A Pearson correlation analysis was conducted using IBM[®] SPSS[®] software version 25 (IBM Corp., New York).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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