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Research Article

In Vitro Study of *Garcinia dulcis* (Roxb.) Kurz Leaves Extract against Hepatitis C Virus

Dadan Ramadhan Apriyanto [1]*, Sri Hartati [2], Tutik Sri Wahyuni [3,4], Maria Inge Lusida [4]

[1] Department of Parasitology, Immunology, and Microbiology, Faculty of Medicine, Universitas Swadaya Gunung Jati, Cirebon 45132, Indonesia
[2] Research Center for Chemistry, Indonesian Institute of Sciences (LIPI), Puspiptek, Serpong 15310, Indonesia
[3] Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia
[4] Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya 60115, Indonesia

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**Corresponding author:*
E-mail: dadanramadhanapriyanto95@gmail.com

ABSTRACT

Garcinia dulcis is a plant commonly used in traditional medicine in tropical regions. It has anti-bacterial, anti-inflammatory, anti-tumor, and anti-malaria properties. Despite its potential benefits, studies on its effectiveness as an antiviral are limited. This study investigated the antiviral properties of *G. dulcis* leaf extract against the JFH-1 strain genotype 2a as a treatment for Hepatitis C Virus (HCV). Huh7it-1 cells infected with the HCV genotype 2a strain JFH-1 were used to determine the antiviral effect of methanol extracts of *G. dulcis* leaves (GD-LE). GD-LE antiviral activity was investigated using a focus-forming assay for anti-HCV and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay for cytotoxicity. A time-of-addition study of anti-HCV was also performed to determine the mode of action of GD-LE. The results showed that GD-LE inhibited HCV replication with an IC_{50} of 17.06 µg/mL without giving any toxicity to the cells. The mode-of-action of GD-LE was found to inhibit HCV replication in the co-addition and post-infection stages. These findings suggest that GD-LE could be a promising candidate for anti-HCV treatment. However, further study regarding GD-LE bioactive compound isolation needs to be done to prepare an effective antiviral.

Keywords: Garcinia dulcis, hepatitis c virus, antiviral activity

Introduction

Hepatitis C Virus (HCV) is a blood-borne virus, predominantly through intravenous drugs, blood transfusions, and non-compliance with universal precautions in healthcare settings. Infection with HCV may result in long-term damage to the liver, such as cirrhosis and hepatocellular carcinoma, which further lead to death. About 399,000 people worldwide were reported dead from the infection of HCV [1, 2].

HCV is a single-stranded RNA virus that belongs to the Flaviviridae family. Its genome is linear, positively oriented, and encodes both structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [3]. HCV exhibits sequence variation in genetic and different genotypes that are classified into seven genotypes and 67 subtypes [4]. Therefore, HCV's high levels of genetic diversity are limited to vaccine development.

The World Health Organization (WHO) recommends treating all individuals with chronic hepatitis C infection with pan-genotypic direct-acting antivirals (DAAs) for adults, adolescents, and children of more than three years old. These oral treatments are short, have minimal side effects, and can effectively cure most cases of HCV infection, depending on the presence of cirrhosis [5].

This therapy has been proven to significantly reduce the incidence and death rates of HCV

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infections. However, it may have side effects, such as fatigue, headache, nausea, diarrhea, and even drug resistance from polymorphisms in HCV [6]. Therefore, it is crucial to keep developing harmless and affordable drugs for HCV therapy from traditional medicine.

Garcinia dulcis is a plant resource that is believed to have medicinal properties. It is naturally distributed throughout Southeast Asia, including Indonesia, Malaysia, the Philippines, and Thailand. In Indonesia, *G. dulcis*, also known as *Mundu* that is commonly used to treat parotitis, lymphatic conditions, struma, and swelling of the thyroid gland, and finally, providing antiviral properties [7].

Studies on *G. dulcis* have found various secondary metabolites [7] with mostly have shown pharmacological benefits, such as antioxidant activity [8], anticancer activity [9], antibacterial activity [10], and antiviral activity [11]. Specifically, in vitro studies have found that *G. dulcis* leaves can inhibit DENV2 replication [11], indicating the potential for developing antiviral drugs for RNA viruses of the Flaviviridae family [12]. This present study evaluated the anti-HCV activity of *G. dulcis* leaves against JFH-1 strain of HCV.

Material and Methods

Plant material

G. dulcis (Roxb.) Kurz leaves were obtained from the Indonesian Institute of Sciences (LIPI) Research Center for Chemistry, located in Serpong, Indonesia. The plant species has been identified by botanists at LIPI's Botani Research Center for Biology in Cibinong, Indonesia, and comes with certificate number 1165/IPH.1.02/IF.8/VII/2014.

Preparation of G. dulcis extract

The extraction method was performed according to Apriyanto *et al.* [13]. To create powdered *G. dulcis*, 1 kg of leaves was dried then extracted using 4 L of methanol for four cycles under reflux conditions. The extract's solution were combined and placed under a vacuum rotary evaporator at 40 ℃ until finally GD-LE was obtained. GD-LE was then made into a stock solution of 100 mg/mL by dissolving it into dimethyl sulfoxide (DMSO) and stored at a temperature of -30 ℃.

Cells culture and viruses preparation

The cells used in this study were cloned from cell lines derived from human hepatocellular carcinoma (Huh7it-1) [14]. The cells were cultured in Dulbecco-modified Eagle medium supplemented with non-essential amino acids (Gibco-Invitrogen), 10% fetal bovine serum (Biowest), and Kanamycin (Sigma-Aldrich). The cells were kept in a humidifying chamber with 5% CO_2 at 37℃. In addition, the virus used in this study was identified as JFH-1 strain of HCV with genotype 2a [15].

Antiviral activity

The antivirus activity assay was performed according to Apriyanto *et al.* [15]. The cells were exposed to viruses with a multiplicity of infection (MOI) of 0.1 while being treated with GD-LE at concentrations of 40, 20, 10, and 5 µg/mL then incubated at 37 ℃ for two hours. Moreover, the virus was removed by washing the cells, and the cells were incubated with GD-LE for 46 hours at 37 ℃. The culture's supernatants were collected for virus titration as described by Apriyanto *et al.* [15]. A time-of-addition study was conducted using 40 µg/mL GD-LE, either during the inoculation phase or after inoculation until virus ready to harvest.

Cytotoxicity assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay for GD-LE cytotoxicity was conducted according to Aoki et al. [14]. Cells were seeded into 96-well plates at a density of 2.0×10^4 cells/well and treated with GD-LE at concentrations of 160, 80, 40, 20, 10, 5, and 2.5 µg/mL. The cells were then incubated at 37 ℃ for 48 hours. Furthermore, the medium was replaced with an MTT-solution medium, and the cells were further incubated for 4 hours.

Statistical analysis

Data obtained were statistically analyzed using one-way analysis of variance (ANOVA). Results were presented as mean ± SD. Student's two-tailed t-test was used to compare the data between sets. A P-value <0.05 was considered statistically significant.

Results and Discussion

GD-LE inhibits HCV infection

The in vitro study on GD-LE was performed to assess its antiviral activity against HCV at different doses. The results showed that GD-LE could inhibit the HCV infection, with an IC_{50} of

17.06 µg/mL (Figure 1A). Moreover, the cytotoxicity of GD-LE against Huh7it-1 cells resulting in no visible signs of cytotoxicity for cells treated with GD-LE up to 160 µg/mL. The IC_{50} was 485.8 µg/mL (Figure 1B).

Mode-of-action of GD-LE

For the possible inhibitory step in anti-HCV, we performed a time-of-addition study of GD-LE (Figure 2). Cells treated with GD-LE for 2 hours only of virus adsorption (co-addition) and cells treated with GD-LE after-virus adsorption until virus harvest (post-infection). The virus combined with GD-LE was treated in the culture cells for 2 hours. For control group co-addition followed by feeding with a medium containing GD-LE for 46 hours. The results showed that GD-LE (40 µg/mL) exhibited anti-HCV activity in both at the co-addition and post-infection stages, with 67.8% and 43.0% inhibition, respectively.

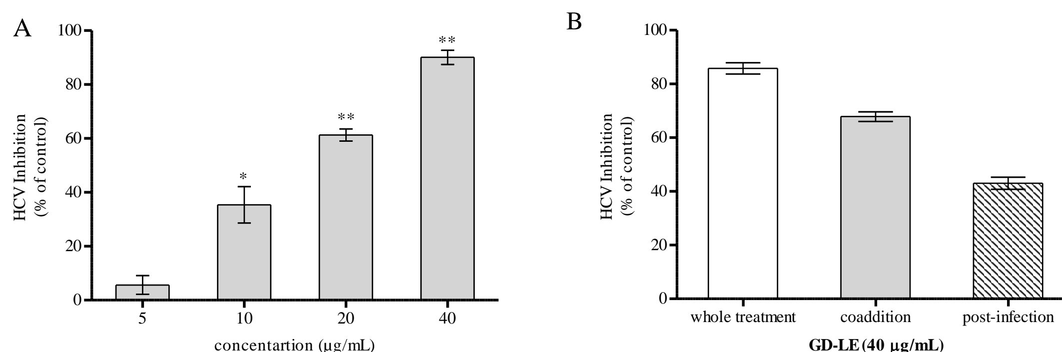

Figure 1. Antiviral and cytotoxicity activity of GD-LE. (A) Inhibition of HCV infection of GD-LE (5 to 40 µg/mL) determined by virus titers in the cell culture (B) Dose-dependent toxicity of GD-LE determined cell viability by MTT assay (2.5 to 160 µg/mL). Data presented as means ± SD of data from triplicate. *, $P < 0.05$; **, $P < 0.01$ compared with the control.

A study conducted on GD-LE against HCV genotype 2a (JFH-1 strain) suggests that it could be a potential candidate as an anti-HCV treatment. Previous research has shown that genus *Garcinia*, specifically the stem bark of *G. celebica* L., has antiviral activity against HCV with an IC_{50} of less than 1.25 µg/mL [16]. Enzymatic mangosteen fruit peel extract has also been found to inhibit RNA helicase HCV [17]. Furthermore, the ethanol extract of the fruit peel from mangosteen (MG-EtOH) has been shown to suppress replication of the HCV genome using sub-genomic of HCV genotype 1b (strain Bart79I) and infectious replicon systems of HCV genotype 2a (strain J6/JFH-1) with EC_{50} values of 5.1 µg/mL and 3.8 µg/mL, respectively [18]. These findings suggest that plants from the *Garcinia* genus may contribute to the resources of plants as anti-HCV treatments.

Medicinal plants contain both secondary metabolites, such as flavonoids, alkaloids, coumarins, and polyphenol compounds, and primary metabolites, such as peptides. These compounds have been reported to possess antiviral effects, including anti-HCV activities [19]. *Garcinia* plants have numerous secondary metabolites found in different parts of the plant. These metabolites exhibit various biological activities which have the potential to cure various diseases [7]. For instance, the leaves of *G. dulcis* are rich in flavonoids, chromones, xanthones, and triterpenoids [20].

The flavonoid compounds found in *G. dulcis* leaves are included in the bioflavonoid group together with morelloflavone, volkensiflavon, GB-2a, amentoflavone, and dulcisbiflavonoid [7]. A study was conducted on biflavonoids from *Garcinia multiflora* leaves, such as GB-1a, GB-2a, volkensiflavone, and morelloflavone, to test their effectiveness against HIV-1 in human lymphocytes. The results showed that morelloflavone had significant antiviral activity against HIV-1 (strain LAV-1) in phytohemagglutinin-stimulated primary human peripheral blood mononuclear cells, with an EC_{50} value of 6.9 µM [21]. In another study, three biflavonoids derived from morelloflavone found in *Dacrydium balansae* leaves and appeared to be powerful inhibitors against DENV-NS5 RdRp with the IC_{50} ranged from 0.26 to 3.12 µM [22].

The leaves of *G. dulcis* contain triterpenoid compounds, such as friedelin [7, 23]. This compound is not exclusive to *G. dulcis* plants because it can be found in other plants. Friedelin extracted

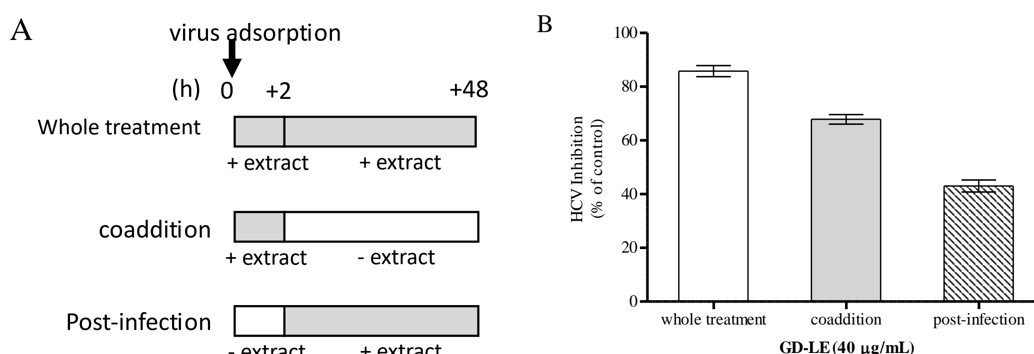

Figure 2. A time-of-addition study of GD-LE. (A) Schematic representation of the time-of-addition experiment. (B) Cells were treated with 40 µg/mL of GD-LE for 48 hours during and after virus adsorption (whole treatment), for 2 hours only during viral inoculation (co-addition), or for 46 hours only after viral inoculation (post-infection). Data presented as means ± SD of data from triplicate.

from *Azima tetracantha* Lam. leaves has antioxidant and liver-protective effects [24], thus exhibits antivirus activity [25, 26]. An in silico study discovered that friedelin from *Clerodendrum* spp. leaves can bind to the SARS-CoV-2 virus spike protein with a binding energy of -42.22 kcal/mol [27]. Another study revealed that friedelin from *Euphorbia neriifolia* leaves has more potent antiviral activity than Actinomycin D, which was used a positive control. The activity of these compounds against human coronavirus (HCoV-229E) was compared to Actinomycin D at 0.02 g/ml, and the percentage of cell survival was compared to a non-treated control at 5 mg/ml. The results showed that friedelin had a cell survival rate of 109%, 3-β-friedelinol had a rate of 132%, and Actinomycin D had a rate of 69.5% [28].

According to this present study, GD-LE can predict how the inhibition process works at different stages of the HCV life cycle. The cycle can be divided into three main phases: (1) virus entry and attachment to target cells, (2) replication and processing of viral proteins and genome, and (3) release and assembly of viral particles. A time-of-addition analysis was conducted to understand how GD-LE works against HCV. The results showed that GD-LE can prevent HCV infection during the entry and post-entry stages. GD-LE may hinder HCV entry by inhibiting direct virucidal effects or viral adsorption. In addition, GD-LE may hinder post-infection steps by directly inactivating virions released from infected cells and partially inhibiting virion assembly [15].

This suggests that GD-LE has multiple secondary metabolites that inhibit viruses. Further research is required to confirm this, and isolating compounds may be necessary for developing an effective antiviral agent.

Conclusion

GD-LE inhibited the hepatitis C virus (HCV) at an IC_{50} of 17.06 µg/mL. It was observed that GD-LE inhibited HCV infection at both during the co-addition and post-infection steps. These findings suggest that GD-LE could be a promising candidate for anti-HCV treatment. However, further study regarding GD-LE bioactive compounds isolation need to be done for preparing an effective antiviral.

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