Original Article

Anti-Hepatitis C Virus Activity of a Crude Extract from Longan (Dimocarpus longan Lour.) Leaves

Dadan Ramadhan Apriyanto1, Chie Aoki2,4*, Sri Hartati4, Muhammad Hanafi4, Leonardus Broto Sugeng Kardono4, Ade Arsianti3, Melva Louisa1, Tjahjani Mirawati Sudiro1, Beti Ernawati Dewi1, Pratiwi Sudarmono1, Amin Soebandrio1, and Hak Hotta4

1Department of Microbiology; 2Department of Chemistry; 3Department of Pharmacology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia; and 4Division of Microbiology, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan

SUMMARY: Infection with hepatitis C virus (HCV) results in hepatitis C, a disease characterized by chronic infection, cirrhosis, and hepatocellular carcinoma. Currently, the standard therapy is a combination of pegylated interferon-α plus ribavirin with NS3 protease inhibitors. Addition of NS3 protease inhibitors to the standard therapy improves response rates; however, use of NS3 protease inhibitors is also associated with significant adverse effects and an increase in the overall cost of treatment. Therefore, there is a need to develop safe and inexpensive drugs for the treatment of HCV infections. In this study, we examined the antiviral activity of a crude extract from Dimocarpus longan leaves against HCV (genotype 2a strain JFH1). The D. longan crude extract (DL-CE) exhibited anti-HCV activity with a 50% effective concentration (EC50) of 19.4 μg/ml without cytotoxicity. A time-of-addition study demonstrated that DL-CE has anti-HCV activity at both the entry and post-entry steps and markedly blocks the viral entry step through direct virucidal activity with marginal inhibition of virion assembly. Co-treatment of DL-CE with cyclosporine A, an immunosuppressant or telaprevir, an NS3 protease inhibitor, resulted in additive and synergistic antiviral effects, respectively. Our findings suggest that DL-CE may be useful as an add-on therapy candidate for treating HCV infections.

INTRODUCTION

Hepatitis virus C (HCV) is a small, enveloped virus belonging to the Hepacivirus genus of the Flaviviridae family. The HCV genome is a single-stranded, positive-sense RNA of 9.6 kb in length and encodes for a single open reading frame (1). The single open reading frame is translated by an internal ribosome entry site located in the 5'-untranslated region (5'UTR) and processed by host peptidases and viral-encoding proteases into 10 polypeptides, including 3 structural (core, E1, and E2) and 7 nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (2). The life cycle of HCV can be divided into 3 major steps as follows, i) entry of the virus into its target cells by receptor-mediated endocytosis; ii) cytoplasmic and membrane-associated replication of the RNA genome; and iii) assembly and release of the progeny virions.

HCV is an important human pathogen infecting approximately 170–200 million people worldwide (3) and contributing to 3–4 million new infections each year (4). HCV infections can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which are a major cause of mortality. HCV is commonly transmitted from infected blood and organ transplants (5). HCV isolates are classified into 7 major genotypes and more than 100 subtypes (6). The distribution of HCV genotypes varies geographically: HCV genotypes 1 to 3 are distributed worldwide, and genotypes 1b and 2a are most common in Asia, including Japan and Indonesia (7–9).

The diversity of HCV isolates hampers vaccine development. Combination therapy using pegylated interferon-α (PEG-IFNα) and ribavirin has been the initial treatment of choice, resulting in improvement in the sustained virological response (SVR); however, SVR rates remain less than 50% in patients infected with genotype 1 HCV (1). To date, many anti-HCV compounds that have been investigated are classified into 2 main classes, direct-acting antivirals (DAAs) and host-targeting antivirals. DAAs target the NS3 protease, NS5A protein, and NS5B RNA polymerase. Host-targeting antivirals target host proteins critical for virus replication, such as cyclophilin A and micro RNA122 (10). Recently, the US Food and Drug Administration approved several NS3 protease inhibitors (e.g., telaprevir, boceprevir) for the treatment of patients infected with HCV genotype 1. The addition of telaprevir to PEG-IFNα plus ribavirin-based therapy decreased drug resistance and increased SVR rates up to 75% in patients with HCV genotype 1 infections (11–13). However, this combination therapy is very expensive and may result in significant adverse effects (14,15). Thus, there remains a need to develop safe, inexpensive, and well-tolerated drugs for the treatment of HCV infections.
Medicinal plants are attractive resources for discovering new biologically active natural products to treat many diseases, including infectious diseases (16,17). During the past several decades, a great deal of research has been invested in natural products from medicinal plants due to the advantages of lower cost, fewer adverse effects, and high chemical diversity of these biologically active molecules. Many plant phytochemicals, such as flavonoids, terpenoids, lignins, alkaloids, tannins, polyphenolics, coumarins, saponins, and chlorophyllins, are reported to have anti-HCV activity (18–26). Thus, natural products from medicinal plants represent an alternative approach to controlling HCV infections.

In the present study, we found that the Dimocarpus longan crude extract (DL-CE) has anti-HCV activity. We investigated the mechanism of antiviral effects of DL-CE, as well as the effects with cyclosporine A and the NS3 protease inhibitor, telaprevir.

**MATERIALS AND METHODS**

**Cells and viruses:** A clone from a human hepatoma-derived cell line, Huh7it-1 (19), was grown in Dulbecco’s modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), non-essential amino acids (Gibco-Invitrogen) and kanamycin (Sigma-Aldrich, St. Louis, MO, USA). Cultured cells were incubated at 37°C in a 5% CO2 humidified chamber. A cell culture-adapted HCV variant (JFH1 strain of genotype 2a) was propagated as described previously (27,28). An Indonesian strain of dengue virus type 2 (DENV, type 2a) was propagated as described previously (27,28). Cell culture-adapted HCV variant (JFH1 strain of genotype 2) was grown in Dulbecco’s (DENV, DS-18/09 strain) was propagated in mosquito-derived C6/36 cells and African green monkey kidney-derived Vero cells as described previously (29,30).

**Virus titration:** Virus titrations were performed as described previously (19,27). Cells were seeded in 96-well plates and then inoculated with culture supernatants serially diluted in culture medium. After virus adsorption for 4 h at 37°C, cells were incubated in medium containing 0.4% methylcellulose (Sigma-Aldrich) for 40 h. Virus titers were determined by a focus-forming assay as described previously (27,28). Virus-infected cells were stained anti-HCV or anti-DENV serum from human patients followed by horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL, Nagoya, Japan). Enzymatic foci were visualized with the Metal Enhanced DAB Substrate kit (Thermo Fisher Scientific, Rockford, IL, USA). Foci images were captured using an OLYMPUS digital camera DP21 attached to an Olympus CX41 microscope (Olympus, Tokyo, Japan) and counted using a katikati counter (<http://www.vector.co.jp/soft/Win95/art/se347447.html>.

A freeze and thaw method was used to determine intracellular HCV infectivity. In brief, virus-infected cells were washed with phosphate-buffered saline (PBS) and suspended in fresh culture medium. The samples were subjected to 3 cycles of freezing and thawing at −80°C and 37°C, respectively, and then centrifuged at 12,000 × g for 5 min at 4°C to remove cell debris. HCV titers were measured as described above.

**Plant material:** D. longan leaves were obtained from the Research Center for Chemistry, Indonesian Institute of Sciences (LIPI), Serpong, Indonesia. Botanists at the Botanical Research Center for Biology, LIPI, Chibinong, Indonesia, identified the plant species. An herbarium specimen was deposited in the Research Center for Chemistry, LIPI.

**Preparation of a DL-CE:** Dried D. longan leaves (500 g) were ground to powder and extracted (3-times) using methanol (2 L) under reflux conditions. The extracts were combined and concentrated under vacuum at 40°C with a rotavapor to obtain the crude extract. The crude extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and stored at −30°C.

**Antiviral activity assay:** An antiviral activity assay was performed as described previously (19). In brief, Huh7it-1 cells were inoculated with HCV or DENV with a multiplicity of infection (MOI) = 1 in the presence of DL-CE (100, 50, 25, 12.5, 6.25, and 3.125 μg/ml) for 2 h at 37°C. After removing any residual virus by washing, cells were incubated for 46 h with the same extract samples. Cell culture supernatants were collected for virus titration. In time-of-addition studies, cells were treated with 50 μg/ml DL-CE, either during the inoculation period or after inoculation for the remaining culture period until virus harvest.

To evaluate the possible effect of co-treating DL-CE with cyclosporine A (Sigma-Aldrich), or telaprevir (AdooQ BioScience, Irvine, CA, USA), a classical isobologram analysis was performed (31).

**Quantitative real time-polymerase chain reaction (qRT-PCR):** Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. One μg of total RNA was transcribed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) with random primers. The cDNA was amplified using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). PCR was performed using Roche 480 LightCycler II system with specific primers to amplify an NS3 region of the HCV genome, 5'-CTTT GACTCCGTGATCGACT-3' (sense) and 5'-CCCTGT CTTCCTCTACCTG-3' (antisense); human β-actin mRNA, 5'-TGGCACCGACGACATGGA-3' (sense) and 5'-CTAAGCTCATAGTCCGCCTAAGGA-3' (antisense), as described previously (32).

**Western blot analysis:** Immunoblotting was performed as described previously with a slight modification (33,34). Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia; Piscataway, NJ, USA). Membranes were incubated with an HCV NS3-specific mouse monoclonal antibody (clone H23; Abcam, Cambridge, MA, USA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MBL) followed by HRP-conjugated goat anti-mouse immunoglobulin (MBL) as the secondary antibody. Bound antibody complexes were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, Bedford, MA, USA).

**Viral adsorption assay:** Cells were inoculated with HCV (MOI = 3) in the presence or absence of the DL-CE (50 μg/ml) at 4°C for 1 h. The cells were extensively washed with cold PBS and suspended in TRIzol reagent
for total RNA extraction.

**Virucidal activity assay:** The HCV suspension (10⁶ FFU/ml, 75 µl) mixed with an equal volume of DL-CE or heparin (Sigma-Aldrich) was incubated for 2 h at 37°C. Cells were inoculated with a dilution (1,250 times) of the treated virus suspension for 4 h at 37°C. After removing viral inoculum, cells were overlaid with 0.4% methylcellulose-containing medium and incubated for 40 h.

**Plasmid construction:** pFK-SGR-GLuc/JFH1 was generated from the subgenomic replicon pSGR-JFH1, in which the neo gene was replaced with *Gaussia* luciferase gene (GLuc). In brief, an HCV replicon expressing GLuc was constructed by replacing neo gene of plasmid pSGR-JFH1 with the luciferase gene from *Gaussia princeps*. The T7 promoter and the HCV 5’UTR were amplified from pSGR-JFH1 by PCR using the primers GLuc-ATG (5' TTTGC-3') and JFH1-GLuc (5'-ACTTTGACTCCCATT TTGGTTTTTCTTTGAGG-3'). GLuc gene was amplified from pRNAi-hGL (Takara Bio) by PCR using the primers GLuc-ATG (5'-ATGGGAGTCAAAGTTCTG) and GLuc-T (5'-TTATGTCACACCAGGCC CCTT-3'). The two PCR DNA fragments were used as templates for the second round of PCR to combine the 5’UTR with GLuc gene. The second PCR product was digested with EcoRI and ligated with the pSGR-JFH1 vector by digesting with EcoRI and PmeI. After the correct DNA sequences were verified, the generated pSGR-GLuc/JFH1 was double digested with AgeI and EcoRV and inserted into linearized pFK with the same restriction enzyme sites.

**Replication inhibition assay using subgenomic replicon:** Methods for in vitro transcription of HCV RNA and its electroporation into cells have been reported previously (32). SGR-GLuc/JFH1 RNA-transfected cells were seeded into 12-well plates. At 4 h post-electroporation, DL-CE or cyclosporine A was added to the medium, and cells were incubated for 48 h. Portions of the culture medium were collected to measure luciferase activity using *Gaussia* luciferase assay kit (New England Biolabs, Ipswich, MA, USA) using GloMax-96 Microplate Luminometer (Promega, Madison, WI, USA). The luminometer was set to automatically inject 50 µl of the GLuc assay solution with 5 s of delay and 5 s of integration.

**Cytotoxicity assay:** Cytotoxicity of DL-CE against Huh7it-1 cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method. Briefly, cells were seeded in 96-well plates and then treated with various concentrations of DL-CE for 48 h. The MTT assay was performed as described previously (19). The percentage of viable cells was plotted versus the concentration of DL-CE. The concentration of DL-CE that elicited 50% cytotoxicity (CC50) was determined by non-linear regression using GraphPad Prism graphing software (San Diego, CA, USA).

**Data analysis:** Results are expressed as the mean ± SEM. Differences between two data sets were evaluated by Student’s two-tailed t-test. A P-value (P) of <0.05 was considered statistically significant.

**RESULTS**

**DL-CE inhibits HCV infection:** We collected approximately 250 kinds of Indonesian medicinal plants from a botanical garden in LIPI, Serpong, Indonesia and evaluated crude extracts from those plants for anti-HCV activity. We found DL-CE inhibited HCV infection in a dose-dependent manner, with a 50% effective concentration (EC50) of 19.4 µg/ml (Fig. 1A). An MTT assay was used to evaluate cytotoxicity of DL-CE against Huh7it-1 cells. No apparent cytotoxicity was observed in Huh7it-1 cells treated with DL-CE up to 400 µg/ml (CC50 = 681.9 µg/ml) (Fig. 1B). The selectivity index (CC50/EC50) was 35.1.

To determine possible inhibitory step(s) in the viral life cycle, we carried out a time-of-addition study using DL-CE (Fig. 2A). Cells were treated with DL-CE during the 2 h of viral inoculation (coadministration) or during the culture period after viral inoculation until virus har-
Fig. 2. Mode-of-action of DL-CE. (A) Schematic representation of the time-of-addition experiment. (B) Cells were treated with 50 µg/ml of DL-CE for 48 h during and after viral inoculation (whole treatment), for 2 h only during viral inoculation (coaddition), or for 46 h only after viral inoculation (post-infection). At day 1 and day 2 post-infection, extracellular virus infectivity was determined by a focus-forming assay. (C) Cells treated with 50 µg/ml of DL-CE described in (B) were analyzed by western blotting against HCV NS3 or GAPDH as a loading control. (D) Cells were treated with DL-CE for 2 h only before viral inoculation (pretreatment), coaddition, or post-infection as shown above. The percentage of HCV infectivity compared to the untreated control (Control) is shown. Data represent means ± SD of data from triplicate.

vest (post-infection). As a positive control, a mixture of HCV and DL-CE was used to inoculate the cells for 2 h, cells were then re-fed with fresh medium containing DL-CE for 46 h (whole treatment). DL-CE (50 µg/ml) showed anti-HCV activity at both the entry and post-entry steps (Fig. 2B), with 75.2% and 90.8% inhibition, respectively. The levels of intracellular HCV NS3 protein accumulation were examined by western blotting analysis. In contrast to the results of infectivity in supernatants, reductions in HCV NS3 protein accumulation after treatment were not clearly observed (Fig. 2C). In addition, in cells pretreated with DL-CE for 2 h before viral inoculation, no effects on HCV infection in the cells were observed (Fig. 2D).

**DL-CE exerts anti-HCV activity through a direct virucidal effect and by partial inhibition of the virion assembly:** The time-of-addition analysis showed that DL-CE has anti-HCV activity at both the entry and post-entry steps. Thus, we proceeded to determine the mechanism(s) of action of DL-CE by conducting three categorized experiments: (i) a virucidal activity assay; (ii) a viral adsorption assay; and (iii) tests for the post-entry event (virus replication, virus translation, and virion assembly). We first measured the virucidal activity. HCV premixed with DL-CE (50 µg/ml) was incubated for 2 h at 37°C before inoculating the cells with virus at 37°C. The results showed that DL-CE could reduce HCV infectivity by 99.7% (Fig. 3A). Next, we investigated the effect of DL-CE on virus adsorption. Cells were preincubated at 4°C and inoculated with HCV at 4°C for 1 h in the presence of DL-CE. The treatment of cells at 4°C can minimize internalization of the virus into the cells after binding to the specific receptor(s). Subsequently, total RNAs from the cells after viral adsorption were extracted and subjected to qRT-PCR to quantify HCV bound to the cell surface. The results showed that DL-CE exhibited weak anti-adsorption activity and decreased the HCV copy number by 33.8% (Fig. 3B). As a positive control, treatment with heparin (400 µg/ml) blocked virus adsorption by 53.8%.

To explore antiviral effect(s) on the post-entry event, we first evaluated the effect of DL-CE on HCV replication. Cells were infected with HCV and then treated with DL-CE at 50 µg/ml for 46 h, followed by quantification of intracellular HCV RNA levels using qRT-PCR. Treatment with DL-CE at the post-entry step slightly reduced intracellular HCV RNA levels com-
resulted in a 30% decrease in the anti-HCV effect observed with DL-CE alone (Fig. 5A). Co-treatment of DL-CE with cyclosporine A or telaprevir did not increase cytotoxicity (data not shown). Moreover, isobologram analysis demonstrated that the inhibitory effects of co-treating DL-CE with cyclosporine A or telaprevir were additive and synergistic, respectively (Fig. 5B).

**DL-CE inhibits DENV infection:** We further examined the effects of DL-CE against DENV, another RNA virus belonging to the same Flaviviridae family. DL-CE exhibited anti-DENV activity with an EC₅₀ of 40.9 µg/ml at day 2 post-infection (Fig. 6).

**DISCUSSION**

*D. longan*, a member of the Sapindaceae family, is commonly found in tropical and subtropical areas such as China, Thailand, Malaysia, Philippines, and Indonesia. It has been used as a traditional medicinal herb and possesses anti-inflammatory, antibacterial, antifungal, antiviral, antioxidant, and anticancer properties (36–39). Crude extract from *D. longan* aerial parts was reported to exhibit antiviral activity against HIV (39); however, to date, there have been no reports that crude extract from *D. longan* leaves have antiviral activity against HCV or other viruses.

We have not yet isolated the compound(s) responsible for the anti-HCV activity of DL-CE. However, phytochemical analysis showed that DL-CE contains high amounts of triterpenes, tannins, flavonoids, and carbohydrates (data not shown). *D. longan* leaves have been reported to contain eight polyphenolic compounds, including ellagic acid, 3,4-O-dimethyl ellagic acid, (+)-catechin, ethyl gallate, gallic acid, kaempferol, quercetin, and kaempferol-3-O-a-L-rhamnopyranoside (40). Recently, Xue et al. isolated 12 compounds from *D. longan* leaves, including quercetin 3-O-(3′-O-2′-methyl-2′-hydroxyethyl)-β-D-xiloside, quercetin 3-O-(3′-O-2′-methyl-2′-hydroxyethyl)-α-L-rhamnopyranoside, azelion, kaempferol-3-O-a-L-rhamnopyranoside, (−)-epicatechin and proanthocyanidin A-2, friedelin, epifriedelanol, β-amyrin, N-benzoylphenylalanine, β-sitosterol, and daucosterol (41). Ellagic acid and gallic acid, which are ubiquitous tannins, are reported to have anti-HCV activity. Ellagic acid was reported to suppress HCV replication of the genotype 2a replicon by targeting the HCV NS3 protein (42), while gallic acid inhibits HCV at the early viral entry step (43). Catechin and epicatechin have also been reported to exhibit anti-HCV activity. Catechin inhibits virus assembly (44), and epicatechin inhibits virus replication by attenuating the cyclooxygenase-2-dependent signal pathway (45). The flavonoid quercetin is reported to inhibit both HCV replication and HCV virion production (19,44). Quercetin inhibits HCV replication by targeting the cellular heat shock proteins 40 and 70 (46). Quercetin also suppresses HCV NS3/NS4A protease activity (47). The anti-HCV activity of the DL-CE in the present study may reflect the effect(s) of either the principal constituent or of the multiple bioactive compounds described above. Further analysis is required to identify the active molecule(s) responsible for the anti-HCV activity of DL-CE.
Fig. 4. Effect of DL-CE on the post-entry step. (A) HCV infected cells (MOI = 1) were treated with DL-CE for 46 h. HCV RNA copy number in the cells was measured by qRT-PCR analysis. β-actin mRNA was used as an internal control for normalization of the HCV RNA amounts. (B) Cells were transfected with HCV subgenomic replicon RNA. At 4 h post-transfection, culture medium from the transfected cells was changed to medium containing DL-CE or cyclosporine A (CyA). Culture supernatants were collected after 48 h of drug treatment, and the luciferase activity was measured as described in the Materials and Methods section. (C) Lysates from the infected cells described in (A) were subjected to western blotting analysis to detect HCV NS3 or GAPDH as a loading control. The relative amount of HCV NS3/GAPDH in each line is shown. (D) Extracellular and intracellular virus infectivity titers from cells described in (A) were determined. Data represent means ± SD of data from triplicate.

Results of the time-of-addition study demonstrated that DL-CE has anti-HCV activity both at the entry and post-entry steps. DL-CE blocked HCV entry through a direct virucidal effect (Fig. 3A), but only marginally inhibited of HCV RNA replication and HCV protein accumulation (Fig. 4A–C). DL-CE exhibited marginal antiviral effects on the virus assembly (Fig. 4D). These data suggested that the anti-HCV activity of DL-CE is primarily through a direct virucidal effect. In addition, the observed reduction in HCV infectivity at the post-entry step is likely due to direct inactivation of virion released from the infected cells and a partial inhibition of the virion assembly.

The current standard treatment for chronic hepatitis patients with HCV genotype 1 infections is a triple combination regimen using PEG-IFNα plus ribavirin with NS3 protease inhibitors (e.g., telaprevir, simeprevir). Moreover, all oral, IFN-free regimens consisting of an NS3 protease inhibitor (asunaprevir) and an NS5A replication-complex inhibitor (daclatasvir) were recently approved for clinical practice. Clinical guidelines discuss optimizing the use of several DAAs with different mechanisms of action in the HCV life cycle to maximize SVR rates and minimize the risk of developing drug resistance. Here, we demonstrated that co-treatment of DL-CE with cyclosporine A or telaprevir has additive and synergistic antiviral effects, respectively (Fig. 5B). There are some advantages to the medicinal use of DL-CE, such as lower costs, fewer adverse effects, and easier access compared to isolated medical constituents or synthetic medicines. Although the use of DL-CE alone has relatively lower anti-HCV effects, its use as a supplement might increase therapeutic outcomes in combination therapy with IFN-free regimens.

In the present report, we demonstrate that DL-CE has anti-DENV activity (Fig. 6). Dengue is a mosquito-borne viral disease found in tropical and subtropical regions. There are currently no effective vaccines or antiviral drugs available; thus, there is a significant need to develop cheap and effective antiviral agents. Our
data show that DL-CE inhibits DENV infection with an EC50 of 40.9 μg/ml, although it is less effective against DENV than HCV. Because both HCV and DENV are members of the Flaviviridae family, the development of anti-HCV agents can also benefit the search for antivirals against DENV.

In conclusion, the present study demonstrates that DL-CE shows anti-HCV activity primarily by inhibiting the entry step through a direct virucidal effect. Furthermore, DL-CE may be useful in the development of other add-on therapy candidate(s) for HCV infections.

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Conflict of interest None to declare.

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