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Running title: Anti-HCV activity of D. longan crude extract
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SUMMARY:

Hepatitis C is a disease caused by hepatitis C virus (HCV) that causes chronic infection, cirrhosis, and hepatocellular carcinoma. The current standard therapy is a combination of pegylated interferon-α plus ribavirin with NS3 protease inhibitors. Addition of NS3 protease inhibitors increases response rates; however, this addition is associated with significant side effects and an increase in the overall cost of the treatment. Therefore, there remains 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 exhibited anti-HCV activity with a 50% effective concentration (EC$_{50}$) of 19.4 μg/ml without cytotoxicity. A time-of-addition study demonstrated that the crude extract exerts anti-HCV activity at both the entry and post-entry steps. The crude extract markedly blocked viral entry step through a direct virucidal effect with a marginal inhibition of virion assembly. The co-treatment of the crude extract with cyclosporine A or telaprevir, an NS3 protease inhibitor, had additive and synergistic antiviral effects, respectively. Our findings suggest that the *D. longan* crude extract may be a candidate of the add-on therapy for HCV infection.
INTRODUCTION:

Hepatitis C virus (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 ten polypeptides: three structural (core, E1, E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (2). The life cycle of HCV can be divided into three major steps: entry of the virus into its target cells by receptor-mediated endocytosis, cytoplasmic and membrane-associated replication of the RNA genome, and 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 infection can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. These chronic liver diseases are a major cause of mortality. HCV is commonly transmitted from infected blood and organ transplants (5). HCV isolates are classified into seven major genotypes and more than 100 subtypes (6). The distribution of HCV genotypes varies geographically and HCV genotypes 1 to 3 are distributed worldwide. Genotypes 1b and 2a are most common in Asia, including Japan and Indonesia (7-9).

Diversity of HCV isolates hampers the development of vaccine. Combination therapy with a pegylated interferon-α (PEG-IFNα) and ribavirin have been used as the initial treatment of choice. This therapy improved the sustained virological response (SVR); however, the SVR rates are no better than 50% in patients infected with
genotype 1 infections (1). Till date, many anti-HCV compounds have been investigated and those are classified into two main classes: direct-acting antivirals (DAAs) and host targeting antivirals. The DAAs target the NS3 protease, NS5A protein, and NS5B RNA polymerase, while 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 some NS3 protease inhibitors (telaprevir, boceprevir, etc.) for therapy of patients infected with HCV genotype 1. The addition of the telaprevir to PEG-IFNα plus ribavirin-based therapy has a higher barrier to drug resistance and increases SVR rates up to 75% in patients with HCV genotype 1 infections (11-13). However, this combination therapy is very expensive and sometimes causes 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 the discovery of new biologically–active natural products for the treatment of many diseases, including infectious diseases (16, 17). Because natural products from medicinal plants have advantages of lower cost and fewer side effects as well as characteristics of high chemical diversity, researchers have explored active molecules from medicinal plants during the last several decades. Phytochemical contents of plants, such as flavonoids, terpenoids, lignin, alkaloids, tannins, polyphenolics, coumarins, saponins and chlorophyllins, were reported to have anti-HCV activity (18-26). Thus, natural products from medicinal plants are alternative approach to control HCV infection.

In the present study, we found that the crude extract from *Dimocarpus longan* leaves has a potential anti-HCV activity. We investigated the mechanism of
action of the inhibition and also antiviral effect of the crude extract in combination with
cyclosporine A and an NS3 protease inhibitor, telaprevir.

MATERIALS AND METHODS

Cells and viruses: A clone from a human hepatoma-derived cell line, Huh7it-1 (19), were grown in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), non-essential amino acids (Gibco-Invitrogen) and kanamycin (Sigma-Aldrich, St. Louis, MO, USA). Cultured cells were incubated at 37°C in 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, 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 titration was performed as described previously (19, 27). Culture supernatants were serially diluted in culture medium and inoculated to the cells in 96-well plates. After virus adsorption for 4 h at 37°C, the 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 with anti-HCV or anti-DENV human patient’s serum followed by horseradish peroxidase-conjugated goat anti-human IgG (MBL, Nagoya, Japan). Infectious foci were visualized with Metal Enhanced DAB Substrate kit (Thermo Fisher Scientific, Rockford, IL, USA). Foci image was captured using Olympus digital camera DP21 attached to Olympus CKX41 microscope (Olympus, Tokyo, Japan) and counted using katikati counter (http://www.vector.co.jp/soft/win95/art/se347447.html).
To determine intracellular HCV infectivity, freeze-and-thaw experiments were performed. In brief, virus-infected cells were washed with phosphate-buffered saline (PBS) and suspended in the fresh culture medium. The samples were subjected to 3 cycles of freezing and thawing at −80°C and 37°C, respectively, and were centrifuged at 12,000 × g for 5 min at 4°C to remove cell debris. HCV titers were measured as described above.

Antiviral activity assay: Antiviral activity assay was performed as described previously (19). In brief, Huh7it-1 cells were inoculated with HCV or DENV at multiplicity of infection (MOI) of 1 in the presence of D. longan crude extract (100, 50, 25, 12.5, 6.25 and 3.125 μg/ml) for 2 h at 37°C. After removing residual virus by washing, the 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 D. longan crude extract only during viral inoculation or only after inoculation for the remaining culture period until virus harvest.

To evaluate the possible effect of co-treatment effect of the D. longan crude extract with cyclosporin A or telaprevir (AdooQ BioScience, CA, USA), the 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 Co. Ltd., Osaka, Japan) with random primers and cDNA was amplified using SYBR Premix Ex Taq (Takara, Kyoto, Japan). PCR was performed using Roche 480 LightCycler II system using specific primers to amplify an NS3 region of the HCV genome; 5'-CTTTGACTCCGTGATCGACT-3' (sense) and
5’-CCCTGTCTTCCTCTACCTG-3’ (antisense), and human β-actin (ACTB) mRNA; 5’-TGGCACCCAGCACAATGAA-3’ (sense) and 5’-CTAAGTCATAGTCCGCCTAGAAGCA-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) (MBL, Nagoya, Japan) followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (MBL, Nagoya, Japan) as the secondary antibody. Bound antibody complexes were detected with immobilon Western (Millipore, Bedford, MA, USA).

**Viral adsorption assay:** Cells were inoculated with HCV (MOI=3) in the presence or absence of the *D. longan* crude extract (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 (10^6 ffu/ml, 75 µl) mixed with an equal volume of *D. longan* crude extract or heparin (Sigma-Aldrich) was incubated for 2 h at 37°C. Cells were inoculated with a dilution (1250 times) of the treated virus suspension for 4 h at 37°C. After removing viral inoculum, the cells were overlaid with 0.4% methylcellulose-containing medium and incubated for 40 h.
**Plasmid construction:** pFK-SGR-GLuc/JFH1 was generated from subgenomic replicon pSGR-JFH1, in which *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 primers JFH1-*Eco*RI (5'-GGAATTCTAATACGACTCACTATAG-3') and JFH1-GLuc (5'-ACTTTGACTCCCCATTTTGGTTTTTCTTTGAGG-3'). GLuc gene was amplified from pRNAi-hGL (TAKARA Bio Inc., JAPAN) by PCR using primers GLuc-ATG (5'-ATGGGAGTCAAAGTTCTGTTTGC-3') and GLuc-T (5'-TTAGTCACCACCGGCCCT-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 *Eco*RI and ligated with the pSGR-JFH1 vector by digesting with *Eco*RI and *Pmel*. After the correct DNA sequences were verified, the generated pSGR-GLuc/JFH1 was double digested with *Age* I and *Eco*RV, respectively, and put into linearized pFK with 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 a 12-well plate. At 4 h post-electroporation, *D. longan* crude extract or cyclosporin A (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium, and the cells were incubated for 48 h. Portions of the culture medium were collected for luciferase assay and luciferase activities were measured with a *Gaussia* luciferase assay kit (New England Biolabs, Ipswich, MA, USA) using a GloMax-96 Microplate Luminometer (Promega, Madison, WI, USA). The luminometer was set to automatically inject 50 µl
of the Gluc assay solution with the following parameters, 5 seconds of delay and 5 seconds of integration.

**Cytotoxicity assay:** Cytotoxicity of *D. longan* crude extract against Huh7it-1 cells was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method. Briefly, cells seeded in 96-well plates were treated with various concentrations of the crude extract for 48 h. The MTT assay was performed as described previously (19). The percentage of viable cells was plotted versus the concentration of crude extract. The concentration by which to mediate 50% cytotoxicity (CC$_{50}$) was determined by non-linear regression analysis using GraphPad Prism graphing software.

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

**Preparation of a crude extract from *D. longan* leaves:** 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 vaccum at 40°C using 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.

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

**Crude extract from *D. longan* 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 that a crude extract from *D. longan* leaves inhibited HCV infection in a dose-dependent manner, with a 50% effective concentration (EC$_{50}$) of 19.4 µg/ml (Fig. 1A). The cytotoxicity of the crude extract against Huh7it-1 cells was examined by an MTT assay. No apparent cytotoxicity was observed when Huh7it-1 cells were treated with *D. longan* crude extract up to 400 µg/ml (CC$_{50}$=681.9 µg/ml) (Fig. 1B). The selectivity index (SI: CC$_{50}$/EC$_{50}$) was 35.1.

To determine the possible inhibition step(s) in the viral life cycle, we carried out a time-of-drug addition study. Cells were treated with *D. longan* crude extract only during 2 h of viral inoculation (entry step) or only after viral inoculation for the remaining culture period until virus harvest (post-entry step). As a positive control, HCV mixed with the crude extract was inoculated to the cells, and after viral inoculation for 2 h, cells were re-fed with fresh medium containing the crude extract for 46 h (whole step). *D. longan* crude extract showed anti-HCV activity at both the entry and the post-entry steps (Fig. 2B). The treatment at the entry and the post-entry steps exhibited 75.2% and 90.8% inhibition, respectively, at 50 µg/ml. The levels of intracellular HCV NS3 protein accumulation were examined by western blotting analysis. In contrast to the results of infectivity in supernatants, the reduction in the amounts of the HCV NS3 protein accumulation by the treatment was not clearly observed (Fig. 2C). In addition, cells were pretreated with the crude extract for 2 h
before viral inoculation. The result showed that the pretreatment of the cells with the crude extract did not affect HCV infection into the cells (Fig. 2D).

**D. longan crude extract exerts anti-HCV activity through a direct virucidal effect and by a partial inhibition of the virion assembly:** The time-of-drug addition analysis showed that *D. longan* crude extract exerts anti-HCV activity at both the entry and the post-entry steps. Thus, we proceeded to determine the mechanism(s) of action of the crude extract 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). Firstly, the virucidal activity was measured. HCV premixed with the crude extract (50 µg/ml) was incubated for 2 h at 37°C before viral inoculation to the cells at 37°C. The result showed that the crude extract could reduce HCV infectivity by 99.7% (Fig. 3A). Next, we investigated the effect of the crude extract on virus adsorption. Cells were preincubated at 4°C and inoculated with HCV at 4°C for 1 h in the presence of the crude extract. The treatment of cells at 4°C can minimize virus internalization into the cells occurring after virus binding to the specific receptor(s). Subsequently, total RNAs from the cells after viral adsorption were extracted and subjected to qRT-PCR to quantify the amount of HCV bound to the cell surface. The result showed that the crude extract exhibited weak anti-adsorption activity and the HCV copy number decreased by 33.8% (Fig. 3B). As a positive control, treatment with heparin (400 µg/ml) blocked the virus adsorption by 53.8%.

Next, to explore antiviral effect(s) on the post-entry event, we first evaluated the effect of the crude extract on HCV replication. Cells were infected with HCV and then treated with the crude extract at 50 µg/ml for 46 h, followed by quantifying intracellular
HCV RNA levels using qRT-PCR. Treatment with the crude extract at the post-entry step slightly reduced intracellular HCV RNA levels compared with the untreated control (15% reduction) (Fig. 4A). A similar result was observed in HCV subgenomic replicon system, which revealed that a treatment with the crude extract causes a 30% decrease of luciferase activity (Fig. 4B). Next, HCV protein accumulation was analyzed by western blotting. In agreement with the effect on the virus RNA replication, treatment with the crude extract hardly affected the levels of HCV NS3 protein accumulation (Fig. 4C); on the other hand, cyclosporin A (2 μg/ml), a positive control inhibitor, reduced HCV NS3 accumulation. Furthermore, to consider the possibilities of defective virion assembly and impaired release of virion, we quantified the amount of intracellular and extracellular infectious viruses. The crude extract at 50 μg/ml reduced the extracellular virus titer from $1.1 \times 10^6$ ffu/ml to $2.2 \times 10^5$ ffu/ml (79.8% reduction) ($P < 0.01$), while the intracellular virus titer from $6.1 \times 10^5$ ffu/ml to $3.3 \times 10^5$ ffu/ml (48.6% reduction) ($P < 0.01$) (Fig. 4D). Taken together, our data suggested that D. longan crude extract exhibits anti-HCV activity through a direct virucidal effect and by a partial inhibition of the virion assembly.

**Co-treatment of a D. longan crude extract with cyclosporine A or telaprevir has additive and synergistic anti-HCV effects, respectively:** Cyclosporine A, a well-known immunosuppressive drug, inhibits HCV replication by mediating the blockage of cyclophilins (35). Telaprevir, an inhibitor of HCV NS3 protease, is used to treat HCV genotype 1 infection in combination with PEG-IFNα plus ribavirin in routine clinical practice. Because we found that the D. longan crude extract inhibits HCV infection, we examined the inhibition effect of the D. longan crude extract in
combination with cyclosporine A or telaprevir. The percent infectivity by co-treatment with cyclosporine A or telaprevir was plotted for fixed concentration of the *D. longan* crude extract. The addition of cyclosporine A or telaprevir resulted in a slight increase of the anti-HCV effect (Fig. 5A). The co-treatment did not increase cytotoxicity (data not shown). Moreover, isobologram analysis demonstrated that inhibitory effect of co-treatment with cyclosporine A or telaprevir was additive and synergistic, respectively (Fig. 5B).

**D. longan crude extract inhibits DENV infection:** We further examined the effect of *D. longan* crude extract against DENV, another RNA virus belonging to the same *Flaviviridae* family. The *D. longan* crude extract exhibited anti-DENV activity with an EC<sub>50</sub> 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, till date there has been no reports that the crude extract from *D. longan* leaves exhibits antiviral activity against HCV and other viruses.

In the present study, we have not yet isolated a compound responsible for the anti-HCV activity of *D. longan* crude extract. Phytochemical analysis showed that the crude extract contains high amounts of triterpenes, tannins, flavonoids, and...
carbohydrates (data not shown). *D. longan* leaves were reported to contain eight polyphenolic compounds: ellagic acid, 3,4-O-dimethyl ellagic acid, (+)-catechin, ethyl gallate, gallic acid, kaempferol, quercetin and kaempferol-3-O-α-L-rhamnoside (40). Recently, Xue et al. reported 12 compounds isolated from *D. longan* leaves; quercetin 3-O-(3″-O-2″-methyl-2″-hydroxylethyl)-β-D-xyloside, quercetin 3-O-(3″-O-2″-methyl-2″-hydroxylethyl)-α-L-rhamnopyranoside, afzelin, kaempferol-3-O-α-L-rhamnopyranoside, (-)-epicatechin and proanthocyanidin A-2, friedelin, epifriedelanol, β-amyrin, N-benzoylphenylalanine-N-benzoylphenylalaninate, β-sitosterol, and daucosterol (41). Ellagic acid and gallic acid, ubiquitous tannins, were reported to exhibit anti-HCV activity. Ellagic acid was reported to suppress HCV replication of genotype 2a replicon by targeting the HCV NS3 protease (42), while gallic acid inhibits HCV at the early viral entry step (43). Catechin and epicatechin were reported to exhibit anti-HCV activity. Catechin inhibits virus assembly (44), and epicatechin inhibits virus replication by attenuating the COX-2-dependent signal pathway (45). The flavonoid quercetin was reported to inhibit HCV replication and HCV virion production (19, 44). Quercetin inhibits HCV replication by targeting the cellular heat shock protein 40 and 70 (46). It also suppresses HCV NS3/NS4A protease activity (47). The anti-HCV activity of the *D. longan* crude extract in the present study may reflect either the effect(s) of the principal constituent or of multiple bioactive compounds described above. Further analysis is required to identify the active molecule(s) responsible for the anti-HCV activity of *D. longan* crude extract.

The result of time-of-drug addition study demonstrated that the *D. longan* crude extract exerts the anti-HCV activity both at the entry and the post-entry steps. The crude extract blocked HCV entry through a direct virucidal effect (Fig. 3A). There was only
marginal inhibition of HCV RNA replication and HCV protein accumulation by the crude extract (Fig. 4A, B and C). The crude extract exhibited marginal antiviral effect on the virus assembly (Fig. 4D). These data suggested that the crude extract exhibits anti-HCV activity primarily through a direct virucidal effect. In addition, the observed reduction in the HCV infectivity at the post-entry step is thought to result from a 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 infection is a triple combination regimen using PEG-IFNa plus ribavirin with NS3 protease inhibitors (telaprevir, simeprevir, etc.). Moreover, all oral, IFN-free regimen consisting of an NS3 protease inhibitor (asunaprevir) and an NS5A replication-complex inhibitor (daclatasvir) was recently approved for clinical practice. Clinical guidelines discuss optimizing the use of several DAAs with a different mechanism of action in the HCV life cycle to maximize SVR rates and minimize the risk of selection of drug resistance. We demonstrated that a co-treatment of D. longan crude extract with cyclosporine A or telaprevir had additive and synergistic antiviral effects, respectively (Fig. 5B). There are some advantages of medicinal use of the crude extract such as less cost, fewer side effects and easier access compared to isolated medical constituents or synthetic medicines. Although the use of D. longan crude extract alone has relatively lower anti-HCV effect, coadministration of the crude extract as a supplement might increase therapeutic outcome in combination therapy with IFN-free regimens.

In the present report, we also demonstrated that D. longan crude extract exhibits anti-DENV activity (Fig. 6). Dengue is a mosquito-borne virus disease and found in tropical and subtropical regions. There is currently no effective vaccine or antiviral drug
available; thus, there is a strong need to develop cheap and effective antivirals. Our data revealed that *D. longan* crude extract inhibits DENV infection with EC$_{50}$ being 40.9 μg/ml, although the effectiveness of the extract against DENV is weaker than the effectiveness against HCV. Because both HCV and DENV are members of the *Flaviviridae* family, an intensive work on anti-HCV antivirals can also benefit the search for antivirals against DENV.

In conclusion, the present study demonstrates that a *D. longan* crude extract exhibits anti-HCV activity primarily by inhibiting the entry step through a direct virucidal effect. The crude extract may be useful to develop a candidate(s) of the add-on therapy for HCV infection.

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**Conflict of interest:** The authors declare no conflict of interests.
REFERENCES:

FIGURE LEGENDS:

Fig. 1: Anti-HCV activity of *D. longan* crude extract. (A) HCV was mixed with serial dilutions of *D. longan* crude extract (12.5 to 100 μg/ml) and inoculated to Huh7it-1 cells at an MOI of 1. After virus adsorption, the cells were incubated with the same concentrations of the crude extract for 48 h. Virus titers from the culture supernatants determined. The percentage of HCV infectivity compared to the untreated control is shown. (B) Huh7it-1 cells were treated with different concentrations of *D. longan* crude extract (12.5 to 800 μg/ml) for 48 h and cell viability was evaluated by MTT assay. The percentage of cell viability compared to the untreated control is shown. Data represent means ± SD of data from triplicate cultures.

Fig. 2: Mode-of-action of *D. longan* crude extract. (A) Schematic representation of the time-of-drug addition experiment. (B) Cells were treated with 50 μg/ml of *D. longan* crude extract 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 the crude extract described in (B) were analyzed by western blotting against HCV NS3 and GAPDH as a loading control. (D) Cells were treated with the crude extract for 2 h only before viral inoculation (pretreatment), for 2 h only during viral inoculation (coaddition), or for 46 h only after viral inoculation (post-infection). The percentage of HCV infectivity compared to the untreated control is shown. Data represent means ± SD of data from triplicate cultures. Control: untreated control.

Fig. 3: Effect of *D. longan* crude extract on virus entry and adsorption steps. (A) Virucidal activity test. HCV suspension was mixed with *D. longan* crude extract (50
μg/ml) or heparin (400 μg/ml), and incubated for 2 h at 37°C. The mixtures were inoculated to the Huh7it-1 cells to determine the remaining virus infectivity. (B) Virus adsorption assay. Cells were inoculated with HCV (MOI=3) in the presence or absence of the *D. longan* crude extract (50 μg/ml) at 4°C for 1 h. After being washed with PBS three times, total cellular RNAs were extracted and HCV RNA copy number was measured by qRT-PCR analysis. β-actin mRNA was used as an internal control for normalization of the HCV RNA amounts. Data represent means ± SD of data from triplicate cultures. Control: untreated control.

Fig. 4: Effect of *D. longan* crude extract on the post-entry step. (A) HCV infected cells (MOI=1) were treated with *D. longan* crude extract 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 the crude extract or cyclosporine A (CycA). 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 and GAPDH as a loading control. The relative amount of HCV NS3/GAPDH in each line was shown. (D) Extracellular and intracellular virus infectivity titers from cells described in (A) were determined. Data represent means ± SD of data from triplicate cultures. Control: untreated control. *, P < 0.005; **, P < 0.001; †, P < 0.000001; #, P < 0.00001, compared with the control.
Fig. 5: Co-treatment effect of a *D. longan* crude extract in combination with cyclosporine A or telaprevir. (A) HCV-infected cells were co-treated with indicated concentrations of *D. longan* crude extract with cyclosporine A or telaprevir. Virus titers at 48 h post-infection were determined. (B) Isobologram analysis. The fixed ratios adjusted by the EC$_{50}$ (FICs) were calculated and the FICs for *D. longan* crude extract, cyclosporine A or telaprevir were plotted. Data represent means ± SD of data from duplicate cultures.

Fig. 6: Anti-DENV activity of *D. longan* crude extract. DENV (DS-18/09 strain) was mixed with serial dilutions of *D. longan* crude extract and inoculated to Huh7it-1 cells (MOI=1). After virus adsorption, the cells were incubated with the same concentrations of the crude extract. Virus titers from the culture supernatants determined. The percentage of HCV infectivity compared to the untreated control is shown. Data represent means ± SD of data from triplicate cultures.
Fig. 1: Anti-HCV activity of *D. longan* crude extract.
Fig. 2: Mode-of-action of *D. longan* crude extract.
Fig. 3: Effect of *D. longan* crude extract on virus entry and adsorption steps.
Fig. 4: Effect of *D. longan* crude extract on the post-entry step.
Fig. 5: Co-treatment effect of *D. longan* crude extract in combination with cyclosporine A or telaprevir.
Fig. 6: Anti-DENV activity of *D. longan* crude extract.