Cladogynos orientalis Zipp. extracts inhibit cell culture−derived hepatitis C virus genotype 2a replication in Huh−7 cells through NS5B inhibition

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ABSTRACT

Objective: To evaluate the potential anti-hepatitis C virus (HCV) activities of *Cladogynos orientalis* Zipp. ex Span and to investigate the molecular mode of action.

Methods: Ethanolic and water extracts from various parts of *Cladogynos orientalis* were examined for cytotoxicity by MTT assay. Sub-cytotoxic concentrations of the extracts were used for further determining anti-HCV activity using cell culture-derived HCV genotype 2a propagated in HepaRG cell line. Immunofluorescence assay was performed to observe the effect on viruses at the pre-entry step. Mode of action at the post-entry step was investigated for the viral RNA and protein expressions by real time RT-PCR and Western blotting assays, respectively.

Results: Although *Cladogynos orientalis* water extracts exhibited lower cytotoxicity than ethanolic extracts, all ethanolic extracts from roots, stems, and leaves of *Cladogynos orientalis* exhibited higher anti-HCV activities than water extracts. The highest anti-HCV activity was observed in infected cells treated with the extracts 5 h after absorption. No extracts showed pre-viral entry effect. At the post-viral entry step, only leaf ethanolic extracts inhibited NS5B expression, while all extracts did not inhibit HCV NS3 expression.

Conclusions: *Cladogynos orientalis* ethanolic extracts could be further studied and the major active compound needs to be identified as a promising source for anti-HCV agents.

1. Introduction

Hepatitis C virus (HCV) is a human pathogen causing chronic liver inflammatory disease. Most HCV infected patients (70%-80%) are chronically infected leading to liver fibrosis, cirrhosis and finally hepatocellular carcinoma[1]. It is estimated that approximately more than 170 million people or 2.5% of the world's population are chronically infected by HCV[2,3]. Currently, HCV infected-liver diseases caused 500 000 people die annually[4]. Moreover, more

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than three million people are infected every year[5,6].

HCV is an envelope, positive sense, single-stranded RNA virus belonging to genus *Hepacivirus* in Faviviridae family. The viral particle diameter is among 40-70 nm and classified into seven major genotypes (1-7) and several subtypes[7]. Each genotype has diversity level of nucleotides more than 30% while subtypes are only 15%[7]. The most prevalent HCV is genotype 1 (49.1%) and genotype 3 (17.9%) with about 137.7 million people infected, followed by genotype 4 and 2[2,8]. At present, there is no vaccine available for prevention of HCV infection[9]. Current therapy of HCV-infected patients is the combination of PEGylated interferon and ribavirin which can reach a sustained virologic response (SVR) only 50% for genotype 1 and 70%-80% for genotype 2[10,11]. In 2011, the United States Food and Drug Administration had approved 2 HCV NS3 protease inhibitor drugs, boceprevir and telaprevir, as direct-acting antiviral drugs (DAAs) for genotype 1. These drugs were used in triple combination with standard therapy and could increase SVR rates up to 70% in genotype 1 infected patient[12,13]. Shortly afterwards in 2013, simeprevir and sofosbuvir, new drugs with effective actions on protease and polymerase inhibitors were approved which showed outcomes of high SVR rates[14,15]. In October 2014, US- Food and Drug Administration approved combination of DAAs that was needless to use interferon or ribavirin to treat HCV infection. Combination of sofosbuvir and ledipasvir has shown 96%-99% SVR rates within 12-14 weeks[16,17]. However, both of interferon and DAAs have many serious side effects and it may lead to HCV resistant problem[18]. In addition, DAAs are very expensive; therefore, some patients suffering from HCV infection could not receive the therapy. Hence, new therapeutic options with low cost and less adverse effect are still urgently required.

Medicinal plants are attractive sources for finding novel anti-HCV agents. *Cladogynos orientalis* (*C. orientalis*) Zipp. ex Span is a plant belonging to the Euphorbiaceae family which is commonly found in Central, Northeast of Thailand and Southeast Asia. Roots of this plant have been used in Thai traditional medicine as carminative and anti-flatulence and recorded in the national list of essential medicines of Thailand. Previous studies reported that ethanolic extract of its leaves showed anti-cytotoxic and anti-apoptosis in hepatocyte cell line (HepG2)[19]. Several parts of this plant possessed antioxidant and antibacterial activities against *Streptococcus suis* and *Staphylococcus intermedius*[20]. Klawikkan and colleagues reported anti-dengue viral activity of *C. orientalis* ethanolic extracts[21]. At present, there is still no report on anti-HCV activity of *C. orientalis*. Therefore, this study was aimed to investigate anti-HCV activity from water and ethanolic extracts of *C. orientalis* by using cell culture-derived-HCV (HCVcc). All extracts were tested for their cytotoxicity by MTT assay and anti-HCV activity was evaluated by focus-formation inhibition test. Moreover, the biologically active extracts were further examined for their mode of actions by real time RT-PCR and Western blotting.

2. Materials and methods

2.1. Cell culture and viruses

HepaRG and Huh-7 cell line were cultured in Dulbecco's Modified Eagle's Medium mixture nutrient F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) containing 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, USA). The cells were maintained at 37 ℃ with 5% CO_2. The plasmid pJFH-1 carrying the full-length cDNA of HCV genotype 2a was kindly provided by Prof. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan).

2.2. Preparation of plant extracts

C. orientalis Zipp. ex Span trees including leaves, stems and roots were collected from Muang District, Nakhon Phanom Province, Thailand. Plant samples were identified by Prof. Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen was deposited at the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand. The extraction method was used as described previously by Sithisarn *et al*[20]. Briefly, the plant was ground and boiled at 80 ℃ with distilled water or refluxed by 75% ethanol for 3 h. Subsequently, the extracts were filtered and slowly evaporated. Crude ethanol and water extracts were dissolved in dimethyl sulfoxide and sterile water, respectively, to make 50 mg/mL stock concentrations before stored at -40 ℃ until used.

2.3. Production of HCV particle–derived cell culture from pJFH–1 (HCVcc)

HepaRG cell line was used to propagate HCVcc, as described previously[18]. Briefly, pJFH-1 was linearized by *Xba* Ⅰ enzyme digestion followed by being *in vitro* transcribed to RNA and transfected to HepaRG cells line by lipofectamine 3000® transfection reagent (Invitrogen, USA) or electroporation (Bio-Rad, USA). The supernatants were harvested 8-10 days post-transfection and cell debris was removed by centrifugation. The virus containing supernatant was precipitated with polyethylene glycol 6000 and concentrated by sucrose gradient ultracentrifugation (Beckman Coulter, USA) at 230 000 rcf for 3 h. The virions were re-suspended in phosphate buffer saline (PBS) containing 3% FBS and stored at -80 ℃ until used.

2.4. Determination of cell viability

Cytotoxicity assay was performed as previously described[21]. Briefly, Huh-7 cells were seeded in 96-well plate at 1×10^4 cells/well

and incubated at 37 ℃ with 5% CO_2 for 24 h. After incubation, the medium was replaced with two-fold serial dilution of plant extracts in culture medium containing 3% FBS and further incubated for 72 h. The untreated culture was used as a negative control. MTT solution was then added 96 h post-infection and further incubated for 2 h. The formazan crystal was dissolved by HCl in isopropanol. Cell viability was measured by spectrophotometer (Tecan, Switzerland) at 570 nm.

For evaluations of anti-HCV activity, the HCVcc at multiplicity of infection (MOI) of 0.5 and selected concentrations of plant extract were seeded into Huh-7 cells in a 96-well plate and further incubated for 72 h. After incubation, MTT solution was added and further incubated for 2 h. The formazan crystal was dissolved by HCl in isopropanol. Cell viability was measured by spectrophotometer (Tecan, Switzerland) at 570 nm. Ribavirin (Sigma, USA), interferon alpha 2a (Biosidus, Argentina) and sofosbuvir (ApexBio, USA) were used as positive controls.

2.5. Determination of the effect on viruses at the pre−entry step

Huh-7 cells were seeded in a 8-well slide chamber and incubated with plant extracts or heparin (positive control; Sigma, USA) for 2 h. After removing the extract, HCVcc at MOI of 0.1 were added and allowed to absorb for 2 h at room temperature before replaced with medium containing 1% methylcellulose. After ten days of post-infection, HCV titer was examined by immunofluorescence assay. Briefly, Huh-7 cells were seeded in a 8-well chamber slide at 3×10^4 cells/well and inoculated with HCVcc supernatant. After 2 h post-absorption, the residual viruses were removed and replaced with complete medium containing 1% methylcellulose (Sigma-Aldrich, USA) before further incubated for 10 d. After incubation, infected cells were fixed with 4% paraformaldehydes (w/v) and permeabilized with 0.2 % Triton-X100 in PBS (v/v). Then, cells were blocked with PBS containing 0.1% tween 20, 1% bovine serum albumin and 3% normal goat serum. After blocking, cells were incubated with primary antibody, anti-HCV core antibody (Abcam, UK,) or anti HCV NS3 antibody (Santa Cruz Biotechnology, USA) at 4 ℃ overnight. After washing with PBS, the secondary antibody, Alexa Fluor® 488-conjugated goat-anti mouse IgG (Life Technologies, USA) was added and further incubated for 1 h. Cell nuclei were stained with DAPI solution (Thermo Fisher Scientific, USA) and visualized under Nikon Eclipse E800 microscopy (Nikon, Japan). The viral infectivity was determined by measuring the foci and compared with the untreated sample.

2.6. Determination of the level of intracellular HCV RNA

The effect of *C. orientalis* extracts at the post-entry level was further

examined. Since HCV could be observed in the culture supernatant within 24 h post infection[22], the extracts were treated at 0, 2, and 5 h post-absorption. Huh-7 cells were inoculated with HCVcc at MOI of 0.1 for 2 h. After removing the virus, 20 μg/mL of plant extracts or positive control (sofosbuvir, ribavirin, or interferon α) were added at 0, 2 and 5 h post-absorption. Infected cells were harvested at 6 days of post-infection. HCV RNA was extracted using QIAamp® Viral RNA mini kit (QIAamp, Germany). Real time RT-PCR was performed by mixing total RNA with KAPA SYBR FAST One-Step qRT-PCR Kits (Kapa Biosystems, USA). The reaction was performed by using Mx3000P qPCR System (Agilent Technologies, Inc., USA) with specific primers for JFH-1 HCV (Forward primer: 5-TCTGCGGAACCGGTGAGTA-3 and Reverse primer: 3-TCAGGCAGTACCACAAGGC-5). The *GAPDH* gene was used as the internal control. The relative RNA was calculated by a standard curve generated from a serial dilution of HCV cDNA or RNA template ranging 10^2-10^8 copies number/reaction.

2.7. Determination of HCV protein expression levels

Huh-7 cell line was infected with HCVcc as described before. Following six days of post-infection, infected cells were harvested and lysed with radioimmunoprecipitation assay buffer. Total proteins were determined by Bradford assay and subjected to 12% polyacrylamide gel electrophoresis. The proteins were blotted into immobilon®-P PVDF Membrane (Merck Millipore, Germany) and blocked with Tris-buffered saline containing 0.05% (v/v) Tween20 and 5% skim milk (w/v). The blotting membrane was incubated with primary antibody (anti-NS3 or anti-NS5B Ab; Santa Cruz Biotechnology, USA) followed by HRP-conjugated anti-mouse IgG antibody. The proteins of interest were visualized by Luminata Forte Western HRP substrate under chemiluminesence detection (Merck Millipore, Germany).

2.8. Statistical analyses

The results were represented by mean ± standard deviation (SD) of triplicate experiments. The data were analyzed by Prism statistical software (GraphPadInc.) and the differences between groups were tested using ANOVA followed by *post−hoc* tests (Tukey's HSD).

3. Results

3.1. Anti−HCV activity of C. orientalis extracts

The extracts of *C. orientalis* were determined for their cytotoxic effects on the human hepatocyte cell lines (Huh-7). The results indicated that *C. orientalis* water extracts exhibited lower cytotoxicity

than ethanolic extracts. The 50% cytotoxicity concentrations (CC_{50}) of all water extracts were more than 1 000 μg/mL, while ethanolic extracts of roots, stems and leaves exhibited CC_{50} at 371.69, 499.72 and 626.46 μg/mL, respectively (Figure 1). Sub-cytotoxic concentrations of the extracts were further used to evaluate anti-HCV activity with HCVcc by MTT assay. Result showed that water extracts exhibited anti-HCV infection at less than 40% (Figure 2). *C. orientalis* ethanolic extracts of roots, stems and leaves inhibited HCVcc infection with 50% inhibitory concentrations (IC_{50}) at 2.38, 3.65, and 17.84 μg/mL, respectively. At 100 μg/mL, the root extract was too toxic and the inhibition percentage could not be calculated (Figure 2).

3.2. Effect of C. orientalis extracts on virus−entry step

From previous experiment, all ethanol extracts exhibited higher HCV inhibitory activity than water extracts. Therefore, the ethanolic extracts of *C. orientalis* were selected to investigate the effect on virus pre-entry step by immunofluorescence assay. All ethanolic extracts possessed weak anti-HCV pre-entry activity with no significant difference ($P>0.05$) while heparin, used as a positive control, efficiently inhibited the viral entry step ($P<0.05$) (Figure 3). This result indicated that ethanolic extracts of *C. orientalis* could not prevent cells from HCV infection.
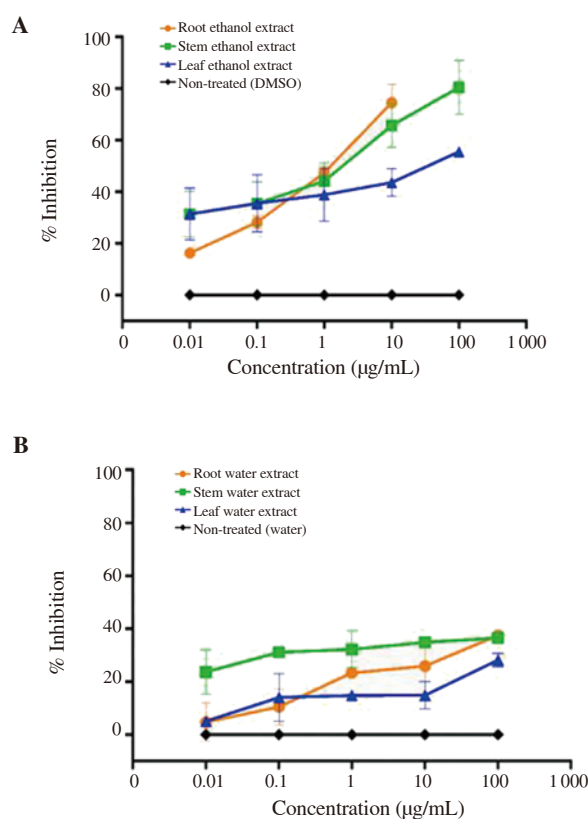

Figure 2. Anti-HCV activity of of ethanol extracts (A) and water extracts (B) of *Cladogynos orientalis* at concentration of 0.01-100 μg/mL. Data represent mean ± SD from triplicate experiment.

3.3. Effect of C. orientalis extracts on intracellular HCV RNA levels

All ethanolic extracts significantly inhibited HCV RNA synthesis at all time intervals except for leaves extracts at 0 h (Figure 4). The root extract showed significant inhibition at 2 h post-absorption. Moreover, all ethanolic extracts exhibited significant HCV-RNA inhibition at 5 h post-HCVcc absorption. This result implied that *C. orientalis* ethanolic extracts could enter the cells to inhibit HCV RNA synthesis.
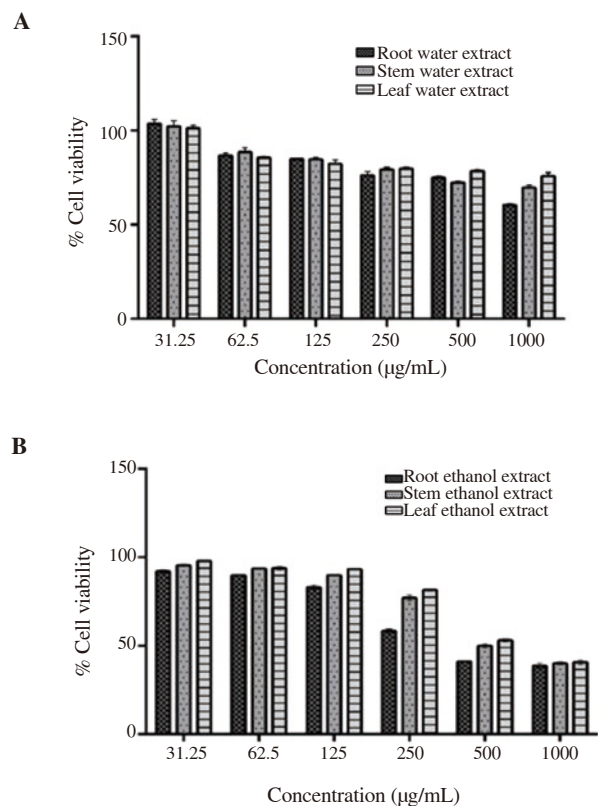

Figure 1. Cytotoxic effect of water extract (A) and ethanol extract (B) of *Cladogynos orientalis*. Data represent mean ± SD from three independent experiments.
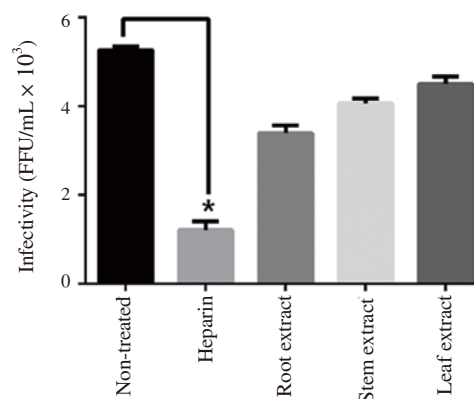

Figure 3. Effect of *Cladogynos orientalis* ethanol extracts on HCV pre-entry. Data represent mean ± SD from triplicate experiment. Statistical analyses were performed using ANOVA followed by *post−hoc* tests (*$P < 0.05$).
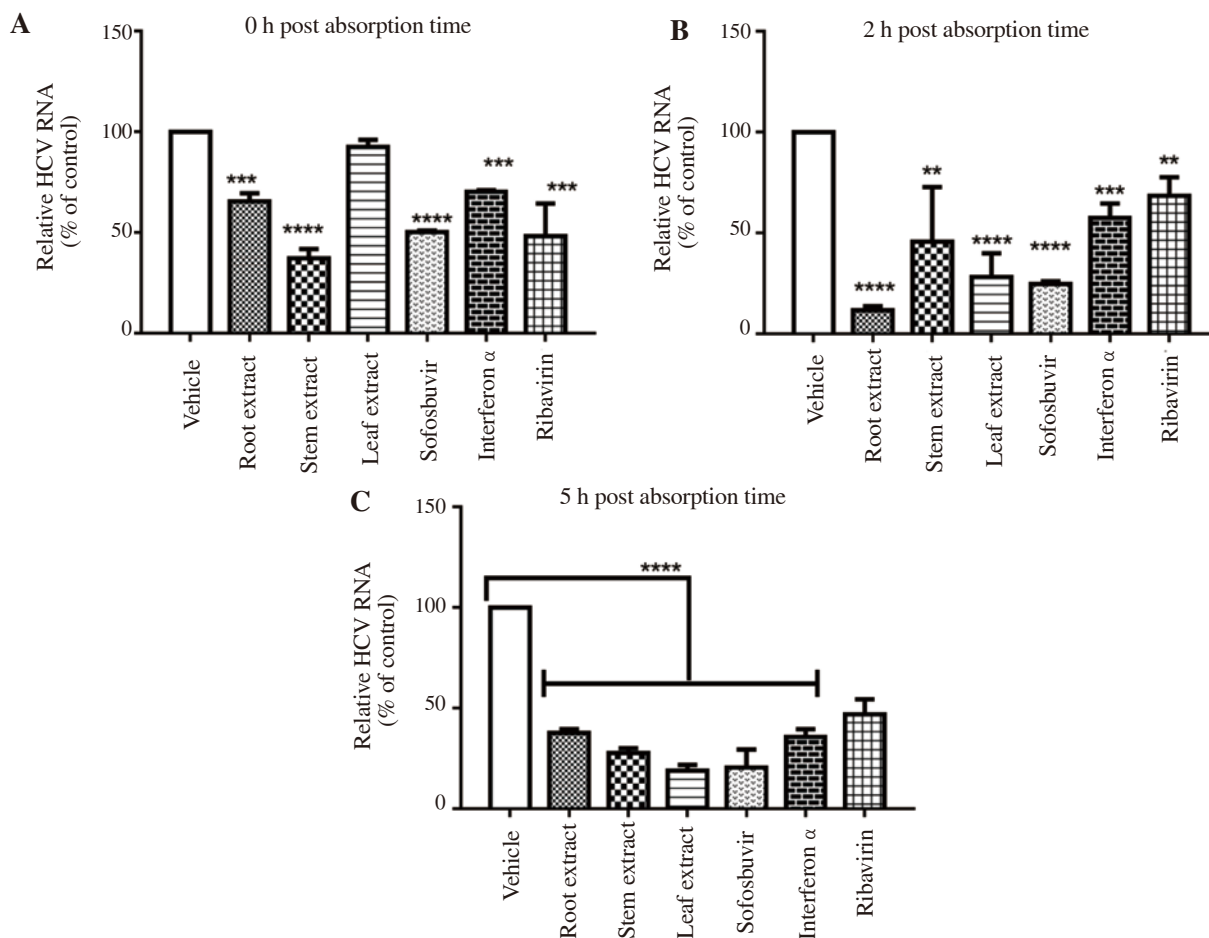
Figure 4. Effect of *Cladogynos orientalis* ethanol extracts on HCV RNA synthesis at 0 (A), 2 (B) and 5 h (C) post infection. Data represent mean ± SD from triplicate experiment (**, *** and **** indicate $P<0.01$, $P<0.001$, and $P<0.000\,1$, respectively).

3.4. Effect of C. orientalis extracts on HCV protein expression levels

The effects of all extracts on HCV protein expressions were further investigated focusing on HCV NS3 and NS5B proteins, essential proteins for HCV RNA replication and also have been reported to be interesting targets for anti-HCV agents. According to the results, all extracts did not inhibit HCV NS3 expression, while only leaf ethanol extracts inhibited NS5B expression at the same level as IFN- α and ribavirin, used as positive controls (Figure 5). In addition, *C. orientalis* stem and root extracts had no effect on NS3 and NS5B HCV protein expressions, similar to sofosbuvir.
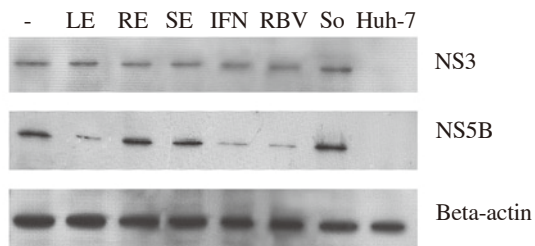

Figure 5. Effect of *Cladogynos orientalis* ethanol extracts on HCV protein expressions. Level of HCV RNA is analyzed by real-time RT-PCR. Expressions of HCV NS3 and NS5B protein are determined by Western blotting with beta-actin as an internal control. LE: leaf extract; RE: root extract; SE: stem extract; IFN: Interferon α ; RBV: Ribavirin; So: Sofosbuvir.

4. Discussion

To date, the development of effective, less toxic and cost-effective anti-HCV agents remains in high demand. Several plants have been extensively studied for their antiviral activity including *Phyllanthus amarus*, *Nymphaea alba* and *Artocarpus heterophyllus*[23–25]. Anti-dengue activity of ethanol extracts of *C. orientalis* Zipp. ex Span. has also been reported[21,26]. In this study, *C. orientalis* extracts were further investigated since dengue virus and HCV belonged to the same family. To study the potentiality of this plant on anti-HCV activity, ethanolic and water extracts of leaf, stem, and root were determined for their cytotoxic concentration. The results showed that all ethanol extracts had cytotoxicity in Huh-7 cell lines. Among three parts, root ethanol extract exhibited the highest cytotoxicity while water extracts had no toxic until the concentration up to 1 mg/mL. Therefore, subcytotoxic concentrations of the extracts were further used to evaluate anti HCV activity. Previous reports suggested that infected HCV could induce cytopathic effect *in vitro* which could be determined by MTT assay[27]. In this study, HepaRG HCVcc was used to examine dose-dependent anti-HCV activity of plant extracts. The results indicated that all ethanolic extracts exhibited anti-HCV activity more potent than all water extracts. This could possibly be explained by active compounds of this plant which were more easily

extracted from ethanol than water. Sithisarn *et al*. investigated the phytochemicals from water and ethanol extracts of *C. orientalis* and found that ethanol extracts contained higher numbers of compounds than water extracts[20]. In addition, previous studies suggested that HCV infection induced inflammatory cytokines and chemokines, or enzymes such as COX-2 in liver cells[28]. HCV-infected cells treated with high concentrations of compounds increased cell inflammation leading to cell death. Root ethanol extracts showed the highest cytotoxicity with more active compounds in the roots than in stems and leaves.

The effects of *C. orientalis* ethanolic extracts were further examined for both pre-entry and post-entry steps of HCV replication. It was clearly shown that all ethanolic extracts had no significant effect at the pre-entry step. When the post-entry step was examined, all ethanolic extracts could significantly inhibit HCV RNA synthesis. This result suggested that *C. orientalis* ethanolic extract could enter the cell and act intracellularly to inhibit HCV RNA synthesis. Moreover, leaf ethanol extracts could inhibit the expression of NS5B. Previous report has shown that *C. orientalis* leaves were rich in phenolics and flavonoids such as chlorogenic acid, catechin, epicatechin, caffeic acid, vanillic acid, coumaric acid, quercertin and rutin[19]. Among these, quercetin was the most abundant compound isolated. Recently, Bachmetov and colleagues reported that quercetin could suppress HCV NS3 protease activity[29]. In agreement with this finding, the results from this study suggested that infected cells treated with *C. orientalis* leaves ethanol extracts could inhibit NS3 protease activity affecting the downstream genome by which inhibiting the synthesis of mature viral proteins, NS5A and NS5B. It was noteworthy that quercetin was not presented in stems and roots of *C. orientalis*; therefore, no effect on HCV protein expression was observed from root and stem extracts[30,31]. The results suggested that leaves extracts probably interfered the function of NS5B protein. This phenomenon was frequently found when examined antiviral activity from plant extracts in concordance with previous reports[30].

Infection with HCV is one of the major causes of liver inflammation worldwide. Hepatitis C is a slow acting virus causing a chronic illness that can be managed with a variety of approaches. Although direct acting antiviral drugs are currently effective with SVR rates up to 96%-99%, these drugs are still considered unaffordable and can introduce serious side effects. Researches for developing suitable therapeutics against the viral infection have attracted a lot of attention and the targeted searches of natural antiviral compounds have been ongoing for safer, cheaper and more importantly non-toxic than the chemical alternatives. This study was the first report that demonstrated that ethanolic extracts of *C. orientalis* showed anti-HCV activity at the post-infection step. The mechanisms were by inhibiting viral RNA synthesis and HCV NS5B proteins which were essential for viral replication. Therefore, *C. orientalis* may be useful as an alternative drug for HCV infected patients and it needs further investigation.

Conflict of interest statement

Acknowledgments

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