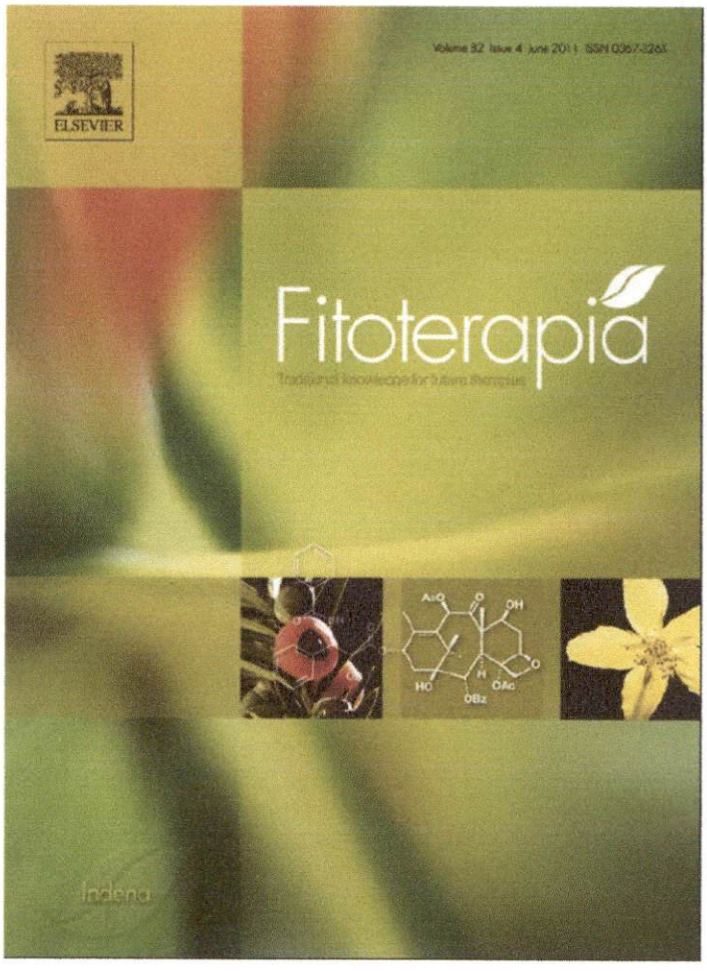

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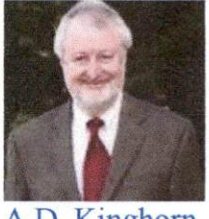

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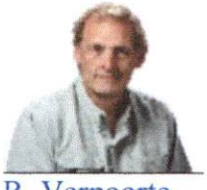

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Inhibition of hepatitis C virus replication by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves

Tutik Sri Wahyuni [a,b], Aty Widyawaruyanti [b], Maria Inge Lusida [c], Achmad Fuad [b], Soetjipto [c], Hiroyuki Fuchino [d], Nobuo Kawahara [d], Yoshitake Hayashi [e], Chie Aoki [a], Hak Hotta [a,*]

[a] Division of Microbiology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[b] Department of Pharmacognocy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Jl. Dharmawangsa Dalam, Surabaya 60286, Indonesia
[c] Institute of Tropical Disease, Airlangga University, Jl. Mulyorejo, Surabaya 60115, Indonesia
[d] Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, 1-2 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan
[e] Division of Infectious Disease Pathology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

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ABSTRACT

Hepatitis C virus (HCV) infection is highly prevalent among global populations, with an estimated number of infected patients being 170 million. Approximately 70–80% of patients acutely infected with HCV will progress to chronic liver disease, such as liver cirrhosis and hepatocellular carcinoma, which is a substantial cause of morbidity and mortality worldwide. New therapies for HCV infection have been developed, however, the therapeutic efficacies still need to be improved. Medicinal plants are promising sources for antivirals against HCV. A variety of plants have been tested and proven to be beneficial as antiviral drug candidates against HCV. In this study, we examined extracts, their subfractions and isolated compounds of *Ruta angustifolia* leaves for antiviral activities against HCV in cell culture. We isolated six compounds, chalepin, scopoletin, γ-fagarine, arborinine, kokusaginine and pseudane IX. Among them, chalepin and pseudane IX showed strong anti-HCV activities with 50% inhibitory concentration (IC_{50}) of 1.7 ± 0.5 and 1.4 ± 0.2 μg/ml, respectively, without apparent cytotoxicity. Their anti-HCV activities were stronger than that of ribavirin (2.8 ± 0.4 μg/ml), which has been widely used for the treatment of HCV infection. Mode-of-action analyses revealed that chalepin and pseudane IX inhibited HCV at the post-entry step and decreased the levels of HCV RNA replication and viral protein synthesis. We also observed that arborinine, kokusaginine and γ-fagarine possessed moderate levels of anti-HCV activities with IC_{50} values being 6.4 ± 0.7, 6.4 ± 1.6 and 20.4 ± 0.4 μg/ml, respectively, whereas scopoletin did not exert significant anti-HCV activities at 30 μg/ml.

1. Background

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus within the *Flaviviridae* family. The viral genome is a single-stranded, positive-sense RNA of 9.6 kb with highly structured 5′- and 3′-untranslated regions [1,2]. It encodes a polyprotein precursor consisting of about 3000 amino acid residues, which is cleaved by the host and viral proteases to generate 10 mature proteins, such as core, E1, E2, a putative ion channel p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. The core, E1 and E2 are cleaved off by the signal peptidase and signal peptide peptidase of the host cell and, together with the viral genome, form the virus particles. The E1 and E2 glycoproteins are responsible for binding to a number of different virus receptor molecules on the cell surface, such as scavenger receptor class B type I, CD81, claudin 1 and occludin. On the other hand, nonstructural proteins play crucial roles in virus replication. NS2 possesses a metalloprotease activity that mediates cleavage between NS2

* Corresponding author. Tel.: +81 78 382 5500; fax: +81 78 382 5519.
E-mail address: hotta@kobe-u.ac.jp (H. Hotta).

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and NS3. After this cleavage takes place, NS3 exerts a serine protease activity that is responsible for the cleavage at the remaining cleavage sites of the polyprotein. NS3 also possesses a helicase activity in its C-terminal domain, which is required for viral RNA replication. NS4A stabilizes NS3 by forming a complex with it and also acts as an inducer of membrane alterations. NS4B is a hydrophobic protein and is involved in the membranous web formation, a characteristic feature of HCV-infected cells. NS5A is a phosphoprotein with versatile functions and is required for viral RNA replication and particle assembly [1,3]. NS5B possesses an RNA-dependent RNA polymerase activity. It is known that the HCV replication cycle is linked to the lipid metabolism of the host cells. It should also be noted that the HCV genome exhibits a considerable degree of sequence heterogeneity, based on which HCV is currently classified into 7 genotypes (1 to 7) and more than 70 subtypes (1a, 1b, 2a, 2b, etc.) [4].

HCV infection is highly prevalent among global populations, especially in Africa and Asia, with an estimated number of infected patients being 170 million worldwide. Approximately 3 million people are newly infected with HCV worldwide every year [3,5]. Seventy to 80% of newly infected patients progress to chronic infection. Patients with chronic HCV infection have a high risk to develop severe liver diseases such as cirrhosis and hepatocellular carcinoma, and also to develop extra hepatic manifestations, including glucose and lipid metabolic disorders [6,7]. A standard care of HCV infection using pegylated interferon (Peg-IFN)-α and ribavirin can achieve sustained virological response (SVR) in ca. 50% of patients infected with HCV genotype 1 or 4 [5]. Triple combination therapy using Peg-IFN-α, ribavirin and an NS3 protease inhibitor increased the SVR rate to 70 to 80%. Moreover, recent approval of other direct-acting antivirals (DAA), including NS5A inhibitors, can further improve the SVR rate. However, they are not equally effective for all of the seven HCV genotypes and, more importantly, serious adverse effects are observed with some patients [5,8]. This highlights the need for a new alternative and/or complementary agent(s) for treatment of HCV.

A wide variety of medicinal plants and their phytochemical constituents have been reported to inhibit HCV infection. For example, an extract of *Phyllantus amarus* root significantly inhibited HCV NS3 protease with a 50% inhibitory concentration (IC_{50}) of 5 μg/ml whereas *P. amarus* leaves inhibited HCV NS5B polymerase with the same IC_{50} value [9]. We tested ethanol extracts of Indonesia plants for their anti-HCV activities and reported that *Toona sureni* leaves, *Melicope latifolia* leaves, *Melanolepis multiglandulosa* stem and *Ficus fistulosa* leaves possessed anti-HCV activities [10]. We also reported that extracts of *Glycyrrhiza uralensis* root and isolated compounds, such as glycycoumarin, glycerin, glycyrol, and liquiritigenin, and extracts of *Morinda citrifolia* leaves, an isolated compound, pheophorbide a, and its related compound, pyropheophorbide a, exhibited anti-HCV activities [11,12]. Likewise, silymarin, iridoid, epigallocatechin-3-gallate were reported to inhibit HCV infection at the entry step while diosgenin, luteolin, quercetin, 3-hydroxy caruilignan C, excoecariphenol D and apigenin at the post-entry step [13,14]. Although a number of novel antivirals against HCV are being developed, further studies are still needed to identify a safer, more effective and cheaper anti-HCV substance(s). Medicinal plants are a good target for the study.

Ruta angustifolia belongs to the Rutaceae family. Plants in the *Ruta* genus have been used as traditional remedy, such as antiseptics, antihelminthics and anti-inflammatory, wound-healing and pain-relief drugs, to cure malconditions during pregnancy and disorders in the gastrointestinal, respiratory, nervous, skin and musculoskeletal systems [15]. In Indonesia, *R. angustifolia* has been known as traditional medicine for liver disease and jaundice. It contains coumarin, alkaloid and flavonoid compounds. Angustifolin and four aromatic derivatives, moskachan A, B, C and D, have been identified as constituents of *R. angustifolia* [16,17]. In this study, we examined the anti-HCV activities of extracts from *R. angustifolia* and its constituents.

2. Materials and methods

2.1. Cells and viruses

Huh7.5 cells and the plasmid pFL-J6/JFH1 to produce the J6/JFH1 strain of HCV genotype 2a [18] were kindly provided by Dr. C. M. Rice, The Rockefeller University, New York, NY, USA. Huh7.5 cells were cultivated in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with fetal bovine serum (Biowest, Nuaille, France), non-essential amino acids (Invitrogen, Carlsbad, CA), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Invitrogen). Cells were grown at 37 °C in a 5% CO_2 incubator.

2.2. Collection, extraction, fractionation and compound isolation of R. angustifolia leaves

R. angustifolia leaves were collected at Lembang, a mountain area of the West Java region, Indonesia. The collected samples were verified by botanical researchers at the Purwadadi Botanical Garden, Purwadadi, Indonesia. Leaves of the plants were dried at room temperature, pulverized and extracted by means of two different extraction procedures; (i) 96% ethanol and (ii) *n*-hexane, dichloromethane and methanol, successively. Maceration process was repeated over 3 days. The obtained filtrates were concentrated under reduced pressure to yield ethanol, *n*-hexane, dichloromethane and methanol extracts. The dichloromethane extract was subjected to the open column chromatography with silica gel (development solvent: gradient of chloroform–methanol system). A bioactivity-positive fraction(s) was further fractionated under open column chromatography with silica gel and mobile phase gradient of hexane–ethyl acetate system. Based on thin layer chromatography (TLC) profiles, several fractions were combined and passed through an activated charcoal column and eluted by each 2 l of methanol (100%), 30% of chloroform–methanol, and chloroform (100%) [19]. Each fraction was concentrated in vacuo and further subfractionated by recycling high-performance liquid chromatography (HPLC) (solvent system: 100% methanol, column: GS-320 + GS-310, 21.5 mm ID × 1000 mm, flow rate: 5.0 ml/min, detection: UV 210 nm) and preparative HPLC (column: Waters XBridge C18 10 × 250 mm, solvent system: gradient solvent of 0.1% trifluoroacetic acid (TFA) — acetonitrile, flow rate: 2.5 ml/min, column temperature: 30 °C). Preparative HPLC was run on JASCO LC-2000 plus series. Recycling preparative HPLC was performed on a Japan Analytical Industry LC-908W.

To determine the structure of the isolated compounds, liquid chromatography–mass spectrometry (LC–MS), ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C NMR analyses were performed. ^{1}H and ^{13}C NMR spectra were measured with BRUKER Ascend 600 spectrometer (600 MHz). LC–MS was performed with a Thermofischer Scientific Orbitrap Elite equipped with an electrospray ionization (ESI) source [20].

2.3. Analysis of anti-HCV activities

R. angustifolia extracts and isolated compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/ml. The stock solutions were stored at −20 °C until used. Ribavirin was purchased (Sigma-Aldrich, Steinheim, Germany) and used as a positive control.

Huh7.5 cells were seeded in 24-well plates (1.9×10^5 cells/well). A fixed amount of the J6/JFH1-P47 strain of HCV genotype 2a [10,21], with a multiplication of infection (MOI) of 0.5 focus-forming units (ffu)/cell, was mixed with serial dilutions of the extracts (100, 30, 10, 1 and 0.1 μg/ml) and compounds (30, 10, 3, 1 and 0.1 μg/ml), and inoculated to the cells. After 2 h, the cells were washed with medium to remove the residual virus and further incubated in the medium containing the same concentrations of the test samples as those during virus inoculation.

Time-of-addition experiments were performed to assess the mode of action of the samples, as described previously [10–12]. In brief, three sets of experiments were done in parallel. (i) To assess the antiviral effect at the entry step, the mixture of HCV and a sample was inoculated to the cells. After virus adsorption for 2 h, the residual virus and the sample were removed, and cells were refed with fresh medium without the sample for 46 h. (ii) To assess the antiviral effect at the post-entry step, HCV was inoculated to the cells in the absence of the sample. After virus adsorption for 2 h, the residual virus was removed and cells were refed with fresh medium containing the sample for 46 h. (iii) As a positive control, HCV mixed with the sample was inoculated to the cells. After virus adsorption for 2 h, the residual virus and the sample were removed, and cells were refed with fresh medium containing the sample for 46 h. Culture supernatants were obtained at 1 and 2 days post-infection (dpi) and titrated for virus infectivity [21]. Virus and cells treated with medium containing 0.1% DMSO served as a control. The percent inhibition of virus infectivity by the samples was calculated by comparing to the control using SPSS probit analysis, and IC_{50} values were determined.

2.4. Real-time quantitative RT-PCR

Total RNA was extracted from the cells using a ReliaPrep RNA cell miniprep system (Promega, Madison, WI) according to the manufacturer's instructions. One μg of total RNA was reverse transcribed using a GoScript Reverse Transcription system (Promega) with random primers and subjected to quantitative real-time PCR analysis using SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) in a MicroAmp 96-well reaction plate and an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA). The primers used to amplify an NS5A region of the HCV genome were 5′-AGACGTATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGCGACGGTGCTGATAG-3′ (antisense). As an internal control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression levels were measured using primers 5′-GCCATCAATGACCCCTTCATT-3′ (sense) and 5′-TCTCGCTCCT GGAAGATGG-3′ (antisense).

2.5. Immunoblotting

Cells were lysed and separated with SDS-polyacrylamide gel electrophoresis as described previously [10,11]. The samples were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), which was then incubated with the respective primary antibodies. The primary antibodies used were mouse monoclonal antibodies against HCV NS3 and GAPDH (Millipore). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Invitrogen) was used to visualize the respective proteins by means of an enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK). The relative band intensity was quantified using densitometry analysis with ImageJ software. The NS3 protein expression levels were normalized to their respective GAPDH protein levels.

2.6. WST-1 assay for cytotoxicity

WST-1 assay was performed as described previously [10]. In brief, Huh7.5 cells in 96-well plates were treated with serial dilutions of the test samples or 0.1% DMSO as a control for 48 h. After the treatment, 10 μl of WST-1 reagent (Roche, Mannheim, Germany) was added to each well and cells were cultured for 4 h. The WST-1 reagent is absorbed by the cells and converted to formazan by mitochondrial dehydrogenases. The amount of formazan, which correlates with the number of living cells, was determined by measuring the absorbance of each well using a microplate reader at 450 nm and 630 nm. Percent cell viability compared to the control was calculated for each dilution of substances and 50% cytotoxic concentration (CC_{50}) values were determined by SPSS probit analysis.

3. Results

3.1. Bioactivity-guided fractionation and purification of extracts from R. angustifolia leaves and isolation of compounds

Dried and pulverized *R. angustifolia* leaves were extracted with ethanol, *n*-hexane, dichloromethane and methanol as described in the Materials and methods section, and examined for antiviral activities against the J6/JFH1-P47 strain of HCV genotype 2a [10,21]. The results revealed that the dichloromethane extract of *R. angustifolia* leaves had potent anti-HCV activity with IC_{50} of 1.6 ± 0.3 μg/ml (Table 1). The dichloromethane extract was further purified by open column chromatography to

Table 1
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of *R. angustifolia* leaves extracts.

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Ethanol extract	3.0 ± 1.4[a]	>100	>30.3
n-Hexane extract	15.6 ± 5.2	>100	>6.4
Dichloromethane extract	1.6 ± 0.3	49.2 ± 3.6	30.8
Methanol extract	8.1 ± 2.0	>100	>12.3

[a] Mean ± SEM of data from two independent experiments.

obtain 6 fractions, of which fraction 4 showed potent anti-HCV activity with IC_{50} of 0.7 μg/ml (Table 2). Fraction 4 was further fractionated under open column chromatography with silica gel and mobile phase gradient of hexane–ethyl acetate system to obtain 29 fractions. Based on the TLC profile, some of the subfractions were combined and 4 groups of subfractions were passed through an activated charcoal column, which were eluted by each 2 l of 100% methanol, 30% chloroform–methanol and 100% chloroform to give 3 fractions (a, 100% methanol; b, 30% chloroform–methanol; c, 100% chloroform) [19]. Each fraction was concentrated in vacuo. Fraction 1a was subjected to recycling HPLC to afford F1a-1 (3.1 mg) and F1a-2 (96.7 mg), which were determined as an identical compound (compound 1). Fraction 2b was separated using preparative HPLC to obtain F2b-1 (compound 2), F2b-2 and F2b-3 (compound 3). Fraction 3c was separated by filtration method with methanol to yield F3c-1 (compound 4) and F3c-2, the latter of which was subjected to preparative HPLC to obtain 3c-2A (compound 5) and 3c-2B (compound 4). Fraction 4a was subjected to preparative HPLC to obtain F4a-3 (compound 6). The structures of the isolated compounds were determined by LC–MS and NMR analyses (Supplementary Information).

Compound 1 (100.7 mg) was isolated as colorless amorphous powder with a molecular formula of $C_{19}H_{22}O_4$ by orbitrap MS, *m/z* 315.15719 $[M + H]^+$ and was identified as chalepin by comparison with NMR literature data [22]. Compound 2 (5.0 mg) was a yellow amorphous powder with a molecular formula of $C_{10}H_9O_4$, *m/z* 193.04833 $[M + H]^+$ and was identified as scopoletin [23]. Compound 3 (3.0 mg) was identified as γ-fagarine [24], an alkaloid compound, which has a molecular formula of $C_{13}H_{12}O_3N$, *m/z* 230.07985 $[M + H]^+$. Compound 4 (28.7 mg) was identified as arborinine [23], another alkaloid compound with a molecular formula of $C_{16}H_{16}O_4N$, *m/z* 286.10952 $[M + H]^+$. Compound 5 (6.5 mg) with a molecular formula of $C_{14}H_{14}O_4N$ was identified as kokusaginine [23,25], an alkaloid compound. Compound 6 (3.7 mg) with a molecular formula of $C_{18}H_{26}ON$, *m/z* 272.20089 was identified as pseudane IX [26]. The structures of the compounds are shown in Fig. 1.

3.2. Anti-HCV activities of the isolated compounds

Anti-HCV activities of the isolated compounds were tested. Ribavirin, which has been widely used for the treatment of HCV infection, was used as a positive control. The results obtained revealed that chalepin (compound 1) and pseudane IX (compound 6) possessed strong anti-HCV activities, with IC_{50} being 1.7 ± 0.5 and 1.4 ± 0.2 μg/ml, respectively (Table 3).

Table 2
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of fractions from a dichloromethane extract of *R. angustifolia* leaves.

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Fraction 1	>100[a]	>100	n.a.[b]
Fraction 2	>100	>100	n.a.[b]
Fraction 3	>100	>100	n.a.[b]
Fraction 4	0.7	42.1	64.8
Fraction 5	>100	>100	n.a.[b]
Fraction 6	7.4	>100	>13.5

[a] Not detectable at the concentration of 100 μg/ml.
[b] Not applicable.

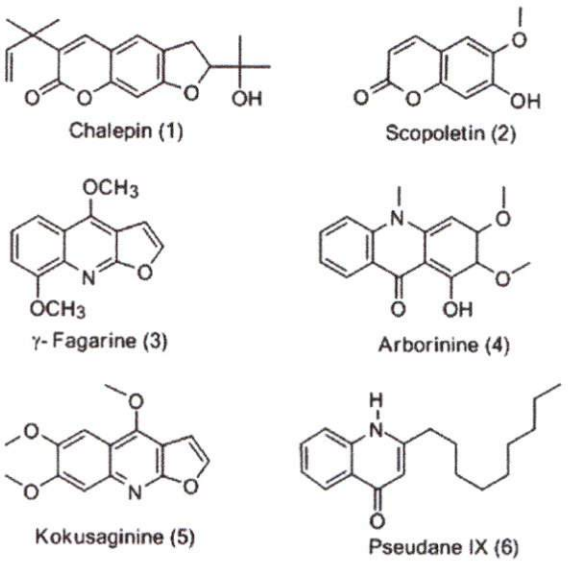

Fig. 1. Molecular structures of the compounds isolated from *R. angustifolia* leaves. Chalepin (compound 1); scopoletin (compound 2); γ-fagarine (compound 3); arborinine (compound 4); kokusaginine (compound 5); pseudane IX (compound 6).

Their anti-HCV activities were stronger than that of ribavirin (2.8 ± 0.4 μg/ml). Also, γ-fagarine (compound 3), arborinine (compound 4) and kokusaginine (compound 5) showed weaker but significant anti-HCV activities, with IC_{50} being 20.4 ± 0.4, 6.4 ± 0.7 and 6.4 ± 1.6 μg/ml, respectively. On the other hand, scopoletin (compound 2) did not show any significant inhibitory effect at the concentration of 30 μg/ml. Dose-dependent profiles of anti-HCV activities and cytotoxicity of those compounds are shown in Fig. 2.

3.3. Mode-of-action of anti-HCV activities of chalepin and pseudane IX

Time-of-addition experiments were performed to determine whether the compounds inhibit HCV at the entry or post-entry step [10]. Percent HCV inhibitions by chalepin and pseudane IX at the concentrations of 30, 10, 3, 1 and 0.1 μg/ml were measured in the experiments where the treatment was done either during (at the entry step), after virus inoculation (at the post-entry step) or both (Fig. 3). The IC_{50} values of chalepin for treatment at the entry step, post-entry step and both were

Table 3
Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of the isolated compounds.

Isolate code	Compound	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Compound 1	Chalepin	1.7 ± 0.5[a]	14.0 ± 2.4	8.2
Compound 2	Scopoletin	>30[b]	>30[b]	n.a.[c]
Compound 3	γ-Fagarine	20.4 ± 0.4	>30[b]	>1.5
Compound 4	Arborinine	6.4 ± 0.7	16.3 ± 6.2	2.5
Compound 5	Kokusaginine	6.4 ± 1.6	>30[b]	>4.7
Compound 6	Pseudane IX	1.4 ± 0.2	26 ± 0.9	18.6
Positive control	Ribavirin	2.8 ± 0.4	>30[b]	>10.7

[a] Mean ± SEM of data from two independent experiments.
[b] Not detectable at the concentration of 30 μg/ml.
[c] Not applicable.

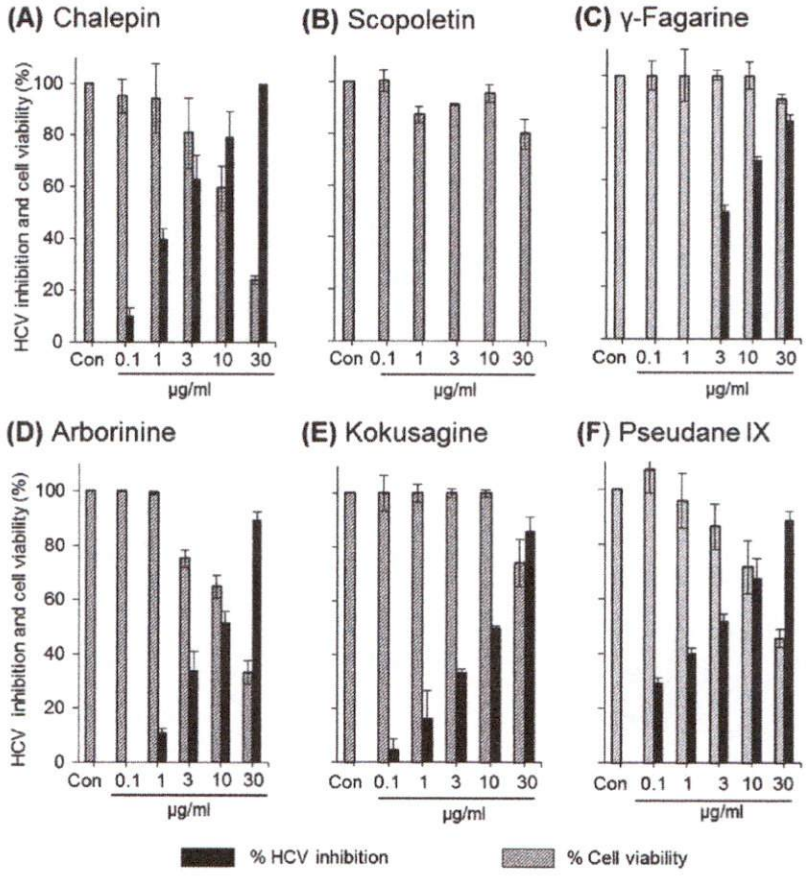

Fig. 2. Dose-dependent inhibition of HCV infection by isolated compounds and their cytotoxicity. Various concentrations of isolated compounds, chalepin (A), scopoletin (B), γ-fagarine (C), arborinine (D), kokusaginine (E) and pseudane IX (F), were mixed with an equal amount of HCV to obtain a final concentration of 30 to 0.1 μg/ml and inoculated to Huh 7.5 cells (MOI = 0.5). After virus adsorption, the cells were cultured with the same concentrations of compounds for 46 h. The culture supernatants were harvested and titrated for virus infectivity. Percent inhibitions of HCV infectivity by each compound are shown. In parallel, cytotoxicity of the compounds was measured by WST-1 assay. Con, control. The data represent means ± SEM of data from two independent experiments.

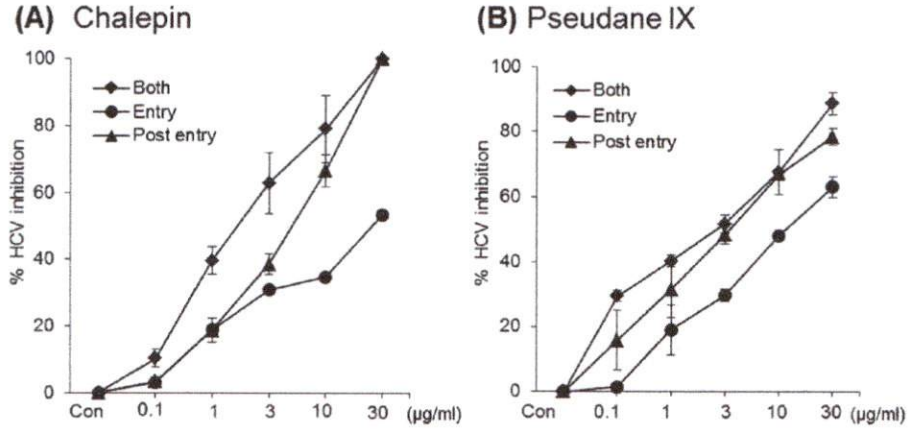

Fig. 3. Mode-of-action analysis of chalepin and pseudane IX. Huh7.5 cells infected with HCV were treated with various concentrations of chalepin (A) and pseudane IX (B) at different timings; (i) only during virus inoculation (entry), (ii) only after virus inoculation (post entry) and (iii) both during and after virus inoculation (both), with a total incubation time of 48 h. Con, control. Percent inhibitions at each concentration and IC_{50} values are shown. The data represent mean ± SEM of data from two independent experiments.

26.7 ± 1.3, 5.2 ± 0.7 and 1.7 ± 0.5 μg/ml, respectively. Also, those for pseudane IX were 11.5 ± 0.2, 3.0 ± 0.9 and 1.4 ± 0.9 μg/ml, respectively. These results suggested that chalepin and pseudane IX inhibited HCV predominantly at the post-entry step.

3.4. Inhibition of HCV RNA replication and HCV protein synthesis by chalepin and pseudane IX

To further confirm that chalepin and pseudane IX inhibit HCV infection at the post-entry step of HCV life cycle, we investigated the effect of those compounds on HCV RNA replication, viral protein synthesis and infectious virus production. Real-time quantitative RT-PCR analysis revealed that chalepin and pseudane IX at 3 and 10 μg/ml inhibited HCV RNA replication (Fig. 4A). Consistently, immunoblotting analysis demonstrated that both compounds inhibited HCV protein synthesis (Fig. 4B). We confirmed in the same experiment that they inhibited HCV production in the culture (Fig. 4C).

4. Discussion

Medicinal plants are good resources to search a novel drug candidate(s). A wide variety of phytochemicals that inhibit virus infections have been isolated and reported. In the present study, we examined the possible anti-HCV activity of *R. angustifolia* extracts and its constituents. *R. angustifolia* is widely distributed throughout the world and has been used as folk medicine for treatment of certain diseases. Plants of the genus *Ruta* have been commonly used

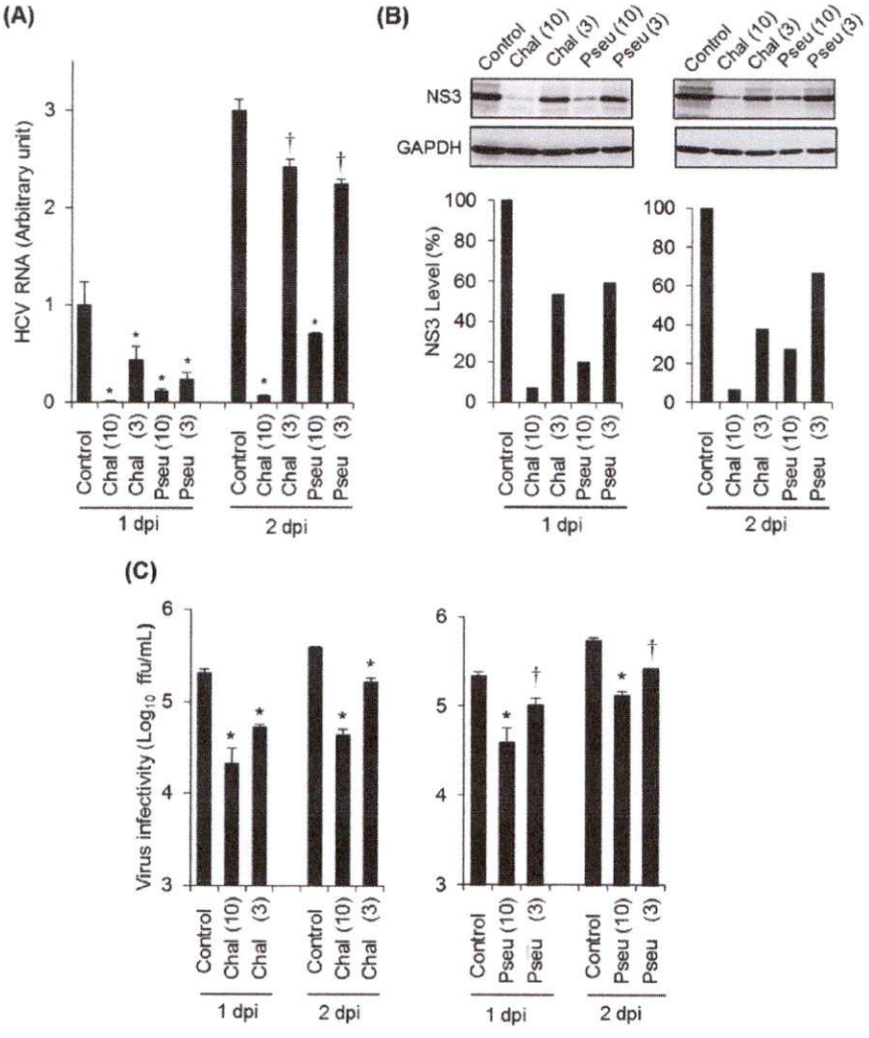

Fig. 4. Inhibition of HCV RNA replication, HCV protein synthesis and infectious virus production by chalepin and pseudane IX. (A) Huh7.5 cells infected with HCV (MOI = 2) and treated with chalepin or pseudane IX (10 and 3 μg/ml) and the untreated control were subjected to real-time quantitative RT-PCR analysis. HCV RNA levels were normalized to GAPDH mRNA expression levels. Data represent mean ± SEM of data from two independent experiments, and the value for the untreated control at 1 dpi was arbitrarily expressed as 1. Chal, chalepin; Pseu, pseudane. *, $p < 0.001$; †, $p < 0.05$. (B) Levels of HCV NS3 protein synthesis in the cells described in (A) were measured by immunoblotting analysis using anti-NS3 monoclonal antibody at 1 and 2 dpi. GAPDH served as an internal control to verify equal amounts of sample loading. Signal intensities of NS3 were normalized to the corresponding GAPDH signal. (C) Virus infectivity in the culture supernatants of the cells described in (A) was measured. *, $p < 0.001$; †, $p < 0.05$.

in both ancient and modern medicine practices in the Mediterranean region to cure pulmonary diseases, rheumatic diseases and helminthic infections [15,27]. A large number of chemical constituents have been isolated from the genus *Ruta*, such as coumarins, alkaloids, benzoquinones, flavone glycosides, sterols, triterpenoids, acridone alkaloids, stigmasterol, lupeol, 5-methoxyarborinine, 5-hydroxyarborinine, ostruthin, bergapten, psoralen, xanthotoxin, limonoid obacunone, isopimpinellin, integriquinolone, kokusaginine, dictamnine, furoquinolin alkaloid, xanthyletin and xanthoxyletin [28,29]. It was also reported that coumarin compounds are the major constituents of the plants in the Rutaceae family, with about two hundred different coumarins being identified [30,31]. Specifically, four aromatic compounds, such as moskachans A, B, C and D have been identified from *R. angustifolia* [17]. Another study on a chloroform extract of aerial part of *R. angustifolia* identified angustifolin, alkaloid graveolin, scoparone and 6,7,8-trimethoxycoumarin [16].

In this study, *R. angustifolia* leaves were subjected to extraction in different polarities of solvents and antiviral activities of the extracts were examined against the J6/JFH1-P47 strain of HCV. The results revealed that a dichloromethane extract of *R. angustifolia* leaves possessed the most potent activity (Table 1), suggesting that a semi-polar compound(s) extracted by dichloromethane was involved in the anti-HCV activity. We further fractionated the dichloromethane extract to obtain 6 fractions. Anti-HCV assay showed that fraction 4 inhibited HCV with IC_{50} of 0.7 μg/ml without apparent cytotoxicity (Table 2). As the TLC profile of the positive fractions suggested the presence of chlorophyll or its related substance(s) in this fraction (data not show), we used activated-charcoal in column chromatography to remove them. Finally, we isolated six compounds and determined their structures by combination of HPLC, LC–MS and NMR; they were chalepin, scopoletin, γ-fagarine, arborinine, kokusaginine, and pseudane IX. Chalepin and scopoletin are classified as coumarins while the remaining four (γ-fagarine, arborinine, kokusaginine and pseudane IX) are alkaloids.

Chalepin, which has been isolated from *R. chalepensis* [32], *Stauranthus perforates* [33], *Clausena anisata* [34] and *Clausena lansium* [35], belongs to furocoumarin compounds with furan ring fused to the coumarin structure. It was reported to possess antimicrobial activities against *Pseudomonas aeruginosa* and *Trichomonas* as well as anti-coagulant activities [34,35]. However, there has been no report so far regarding its antiviral activity against HCV. To the best of our knowledge, the present study is the first to demonstrate anti-HCV activities of chalepin with IC_{50} being 1.7 ± 0.5 μg/ml (5.4 ± 0.5 μM) (Table 3). It was reported that chalepin inhibited respiration of isolated rat liver mitochondria by 40% at the concentration of 16 μM (5.0 μg/ml) [36]. Under our experimental conditions using cultured cells, chalepin exerted only marginal cytotoxicity, if any, at 30 μg/ml (Fig. 2).

We have reported that other coumarin compounds, such as glycycoumarin, glycyrin and glycyrol, possess anti-HCV activities, with IC_{50} of 8.8, 7.2, and 4.6 μg/ml, respectively [11]. The basic structure (1,2-benzopyron) of coumarin appears to be important for binding to HCV [37]. Fourteen compounds out of 24 coumarin derivatives were reported to inhibit HCV NS5B RNA polymerase activities with IC_{50} values between 17 and 63 μM. The activities of those compounds were influenced by the position of methylation or hydroxylation groups in the benzopyron ring [37]. Recently, benzimidazole derivatives of coumarins have been reported to possess increased inhibitory effect on RNA polymerase of HCV NS5B [38,39]. On the other hand, scopoletin, which is the other coumarin isolated in the present study, did not inhibit HCV at the concentration of 30 μg/ml (155 μM). Scopoletin isolated from several plants, such as *Erycibe obtusifolia* Benth, *Aster tataricus* and *Foeniculum vulgare*, and its synthetic derivatives have been extensively studied [40]. Further detailed analyses of derivatives of chalepin and scopoletin would help us understand the structural basis of anti-HCV activity of chalepin and generate a more potent anti-HCV compound(s).

γ-Fagarine, arborinine and kokusaginine, which are alkaloid compounds, showed moderate inhibition with IC_{50} of 20.4 ± 0.4, 6.4 ± 0.7 and 6.4 ± 1.6 μg/ml, respectively (Table 3). These compounds have been isolated from several plants, including the Rutaceae family [41–44] and arborinine was previously reported to inhibit human rhinovirus with IC_{50} of 3.19 μM by virtual model [45]. Pseudane IX, another alkaloid, also known as 2-nonyl-4 (1H)-quinolone, 2-nonyl-4-hydroxyquinoline (NHQ) or 4-hydroxy-2-nonylquinolone [46], showed potent anti-HCV activities with IC_{50} of 1.4 ± 0.2 μg/ml (5.1 ± 0.2 μM) (Table 3). A wide variety of quinolones have been used as antimicrobial, anticancer and antiallergenic agents. Quinolones are known as broad-spectrum antibacterial agents with the main structure of 1,4 dihydro-4-oxo-quinolinyl moiety. Quinolones inhibit prokaryotic type II topoisomerases through direct binding to bacterial chromosome, and likewise, it may bind to viral nucleic acids and/or nucleoprotein complexes to act as antivirals [47]. Quinolones consist of heterobicyclic aromatic compounds and the moiety of C_9H_{19} at carbon number 2 of pseudane IX may play an important role in its activities. Quinolones have been reported to act as inhibitors of HCV NS5B RNA polymerase by binding to the allosteric site II (non-nucleoside inhibitor-site 2) of this protein [48]. Twelve fluoroquinolone derivatives were reported to inhibit HCV replication and HCV NS3 helicase activity in cultured cells. Among them, fleroxacin, difloxacin, ofloxacin, 8-quinolinol, enoxacin, lumifloxacin and flumiquine were reported to inhibit HCV with IC_{50} of 3.8, 2.5, 2.4, 2.2, 1.0, 1.9 and 1.7 μM, respectively [49].

The key steps in HCV life cycle include entry into the host cells, uncoating and replication of the viral genome, translation of virus proteins, and assembly and release of the virion [8,18]. To determine the impact of active compounds on HCV life cycle, we conducted time-of-addition experiments. We observed that chalepin and pseudane IX exhibited their anti-HCV activities at the post-entry step, inhibiting HCV RNA replication and NS3 protein synthesis.

In conclusion, we have identified chalepin and pseudane IX as anti-HCV compounds. These compounds could be good candidates to develop novel anti-HCV drugs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.10.011.

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