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

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(Degree)
博士（医学）

(Date of Degree)
2017-03-25

(Resource Type)
doctoral thesis

(Report Number)
甲第6771号

(URL)
https://hdl.handle.net/20.500.14094/D1006771

（課程博士関係）

学 位 論 文 の 内 容 要 旨

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

Ruta angustifolia の葉から単離された chalepin と pseudane IX による HCV 複製の阻害

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INTRODUCTION

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. It encodes a polyprotein precursor consisting of about 3,000 amino acid residues, which is cleaved by the host and viral proteases to generate 10 proteins, such as core, E1, E2, a putative ion channel p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B.

HCV infection is highly prevalent among global populations, with an estimated number of infected patients being 170 million worldwide. Patients with chronic infection have a high risk to develop severe liver diseases such as cirrhosis and hepatocellular carcinoma. New therapies for HCV infection have been developed, however, the therapeutic efficacy still needs to be improved. A wide variety of medicinal plants and their phytochemical constituents have been reported to inhibit HCV infections.

In this study, we analyzed anti-HCV activities of extracts of *Ruta angustifolia* (*Rutaceae*) and its constituents. In Indonesia, *R. angustifolia* has been known as traditional medicine for liver disease and jaundice.

METHODS

Extraction and isolation of *R. angustifolia* leaves.

R. angustifolia leaves were collected at Lembang, the West Java, Indonesia. Leaves of the plants were extracted in two procedures; (i) 96% ethanol and (ii) n-hexane, dichloromethane and methanol, successively. The dichloromethane extract was further fractionated under open column chromatography, high-performance liquid chromatography (HPLC) and preparative HPLC. 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.

Analysis of anti-HCV activities.

Huh7.5 cells were seeded in 24-well plates and infected with the mixture of HCV J6/JFH1 and serial dilutions of the extracts or compounds. After 2 hours, the cells were washed and further incubated in the medium containing the same concentrations of the test samples. 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 hours, the residual virus and the sample were removed, and cells were refed with fresh medium without the sample for 46 hours. (ii) To assess the antiviral effect at the post-entry step, HCV was inoculated to the cells in the absence of the sample for 2 hours, the residual virus was removed and cells were refed with fresh medium containing the sample for 46 hours.

(iii) As a positive control, HCV mixed with the sample was inoculated to the cells. After virus adsorption for 2 hours, the residual virus and the sample were removed, and cells were refed with fresh medium containing the sample for 46 hours. Culture supernatants were obtained at 1 and 2 days post-infection and titrated for virus infectivity.

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).

Immunoblotting.

Cells were lysed and separated with SDS-polyacrylamide gel electrophoresis. Samples were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), which was then incubated with the respective primary antibodies. The NS3 protein expression levels were normalized to their respective GAPDH protein levels.

Cytotoxicity analysis.

Cytotoxicity analysis was performed by WST-1 assay. Huh7.5 cells in 96-well plates were treated with samples for 48 hours. After the treatment, 10 μl of WST-1 reagent (Roche, Mannheim, Germany) was added to each well and cells were cultured for 4 hours.

RESULTS

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

Anti-HCV activity tests revealed that the dichloromethane extract from *R. angustifolia* leaves had the highest potency with IC_{50} of 1.6 ± 0.3 μg/ml. The dichloromethane extract was further purified by open column chromatography to obtain 6 fractions, of which fraction 4 showed potent anti-HCV activity with IC_{50} of 0.7 μg/ml. Fraction 4 was further fractionated under open column chromatography and obtained 29 fractions. Further purifications were performed by an activated charcoal column and recycling HPLC to obtain 6 isolated compounds. The structures of the isolated compounds were determined by LC-MS and NMR analyses. Molecular structure of isolated compounds were identified as chalepin (100.7 mg), scopoletin (5.0 mg), γ-fagarine (3.0 mg), arborinin (28.7 mg), kokusaginin(6.5 mg) and pseudane IX (3.7 mg).

Anti-HCV activities of the isolated compounds.

Anti-HCV activity test of the isolated compounds revealed that chalepin and pseudane IX possessed strong anti-HCV activities, with IC_{50} being 1.7 ± 0.5 and 1.4 ± 0.2 μg/ml, respectively. Also, γ-fagarine, arborinine and kokusaginine 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 did not show any significant inhibitory effect at the concentration of 30 μg/ml.

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

Mode-of-action analysis revealed that chalepin and pseudane IX inhibited HCV predominantly at the post-entry step. The IC_{50} values of chalepin for treatment at the entry step, post-entry step and both were 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.

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

Real-time quantitative RT-PCR analysis revealed that chalepin and pseudane IX at 3 and 10 μg/ml inhibited HCV RNA replication. Consistently, immunoblotting analysis demonstrated that both compounds inhibited HCV protein synthesis. We confirmed in the same experiment that they inhibited HCV production in the culture.

DISCUSSION

Medicinal plants are good resources to search a novel drug candidate(s), including anti-HCV agents. The present study, *R. angustifolia* leaves were extracted in different polarity of solvents and antiviral activities against the J6/JFH1-P47 strain of HCV were examined. The dichloromethane extract of *R. angustifolia* leaves possessed the most potent activity, suggesting that a semi-polar compound(s) extracted by dichloromethane was involved in the anti-HCV activity. Further fractionation, isolation, and structure determination of the dichloromethane extract lead to the isolation of six compounds; 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. chalepin*, *Stauranthus perforates*, *Clausena anisata* and *C. lansium*, 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. 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. The basic structure (1,2-benzopyron) of coumarin appears to be important for binding to HCV. Scopoletin, the other coumarin isolated in the present study, did not inhibit HCV at the concentration of 30 μg/ml (155 μM). Previous studies reported that scopoletin isolated from several plants, such as *Erycibe obtusifolia* Benth, *Aster tataricus* and *Foeniculum vulgare*, showed only a low level of antitumor activity (IC_{50} >100 μM). On the other hand, 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. Another alkaloid, pseudane IX, also known as 2-nonyl-4 (1H)-quinolone, 2-nonyl-4-hydroxyquinoline (NHQ) or 4-hydroxy-2-nonylquinolone, showed potent anti-HCV activities with IC_{50} of 1.4 ± 0.2 μg/ml. A wide variety of quinolones have been used as antimicrobial, anticancer and antiallergenic agents. 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.

In conclusion, chalepin and pseudane IX isolated from *R. angustifolia* leaves exhibit strong anti-HCV activities, predominantly at the post-entry step, inhibiting HCV RNA replication and NS3 protein synthesis. We propose that chalepin and pseudane IX could be good candidates to develop anti-HCV agents.