Fitoterapia 99 (2014) 276–283

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

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

ARTICLE INFO

Article history:
Received 7 August 2014
Accepted in revised form 11 October 2014
Accepted 14 October 2014
Available online 22 October 2014

Chemical compounds studied in this article:
Chalepin (PubChem CID 119066)
Scopoletin (PubChem CID 5280460)
Arborinine (PubChem CID 5281832)
γ-Fagarine (PubChem CID 107936)
Kokusaginine (PubChem CID 10227)
Pseudane IX (PubChem data not available)

Keywords:
Hepatitis C virus
Ruta angustifolia
Rutaceae
Post-entry inhibition
Alkaloid
Coumarin

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).

http://dx.doi.org/10.1016/j.fitote.2014.10.011
0367-326X/

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.