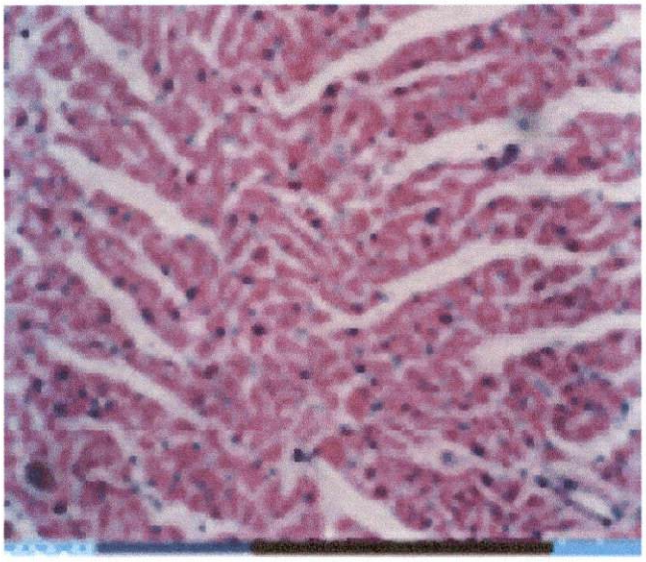

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ORIGINAL ARTICLE

Anti-hepatitis C virus compounds obtained from *Glycyrrhiza uralensis* and other *Glycyrrhiza* species

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ABSTRACT

Development of complementary and/or alternative drugs for treatment of hepatitis C virus (HCV) infection is still much needed from clinical and economic points of view. Antiviral substances obtained from medicinal plants are potentially good targets to study. *Glycyrrhiza uralensis* and *G. glabra* have been commonly used in both traditional and modern medicine. In this study, extracts of *G. uralensis* roots and their components were examined for anti-HCV activity using an HCV cell culture system. It was found that a methanol extract of *G. uralensis* roots and its chloroform fraction possess anti-HCV activity with 50%-inhibitory concentrations (IC_{50}) of 20.0 and 8.0 μg/mL, respectively. Through bioactivity-guided purification and structural analysis, glycycoumarin, glycyrin, glycyrol and liquiritigenin were isolated and identified as anti-HCV compounds, their IC_{50} being 8.8, 7.2, 4.6 and 16.4 μg/mL, respectively. However, glycyrrhizin, the major constituent of *G. uralensis*, and its monoammonium salt, showed only marginal anti-HCV activity. It was also found that licochalcone A and glabridin, known to be exclusive constituents of *G. inflata* and *G. glabra*, respectively, did have anti-HCV activity, their IC_{50} being 2.5 and 6.2 μg/mL, respectively. Another chalcone, isoliquiritigenin, also showed anti-HCV activity, with an IC_{50} of 3.7 μg/mL. Time-of-addition analysis revealed that all *Glycyrrhiza*-derived anti-HCV compounds tested in this study act at the post-entry step. In conclusion, the present results suggest that glycycoumarin, glycyrin, glycyrol and liquiritigenin isolated from *G. uralensis*, as well as isoliquiritigenin, licochalcone A and glabridin, would be good candidates for seed compounds to develop antivirals against HCV.

Key words antiviral substance, coumarin, *Glycyrrhiza uralensis*, hepatitis C virus.

Hepatitis C virus is a member of the genus *Hepacivirus* and the family *Flaviviridae*. Based on the heterogeneity of the viral genome, HCV is currently classified into seven genotypes (1–7) and more than 67 subtypes (1a, 1b, 2a, 2b etc.) (1, 2). The viral genome, a single-stranded, positive-sense RNA of 9.6 kb, encodes a polyprotein precursor consisting of about 3000 amino acid residues that is cleaved by host and viral proteases to generate 10 mature proteins, namely core, E1, E2, a putative ion channel p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (3). Core, E1 and E2 are components of the infectious virus particle together

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List of Abbreviations: CC_{50}, 50%-cytotoxic concentrations; E, envelope; Fr, fraction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; IC_{50}, 50%-inhibitory concentration; ID, internal diameter; SI, selectivity index.

with the viral genome; however, the nonstructural proteins constitute the viral replication complex, where replication of the viral genome takes place. The HCV proteins also play essential roles in the pathological processes associated with HCV infection, such as carcinogenesis and glucose and lipid metabolic disorders (4, 5).

Hepatitis C virus is among the major causative agents of chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (5–7). The global prevalence of HCV is >2.5%; thus, about 180 million people are chronically infected with this virus worldwide. A variety of standard treatment regimens using combinations of pegylated interferon, ribavirin and other direct-acting agents, such as HCV-specific inhibitors against NS3 protease and NS5A, have been adopted with considerable success. However, some clinically important issues remain unsolved, such as the emergence of drug-resistant virus and the cost of these drugs. Therefore, development of complementary and/or alternative drugs, especially those from medicinal plants, for treating HCV infection is still much needed from both clinical and economic points of view (8, 9).

Glycyrrhiza uralensis and *G. glabra* have been widely used as supplementary treatments in both traditional herbal medicine and modern medicine (10, 11). The radix of *Glycyrrhiza* spp. is commonly known as "gan cao" in Chinese and licorice in English. Bioactive constituents of *Glycyrrhiza* species can be classified into triterpenoids (such as glycyrrhizic acid), coumarins (such as glycycoumarin, glycyrin and glycyrol), flavones (such as liquiritin and liquiritigenin), chalcones (such as isoliquiritigenin and licochalcone A), isoflavans (such as glabridin), stilbenoids and other miscellaneous compounds (11). Glycyrrhizic acid, also known as glycyrrhizin and considered the principal component of *Glycyrrhiza* spp., is a glycosylated triterpenoid saponin that consists of one molecule of glycyrrhetinic acid and two molecules of D-glucuronic acid. Upon hydrolysis, the aglycone, 18β-glycyrrhetinic acid (simply called glycyrrhetinic acid), and two molecules of D-glucuronic acid are released. Glycyrrhizin and other compounds isolated from *Glycyrrhiza* species reportedly have antiviral activity against a variety of viruses, including HIV, herpes simplex virus, influenza virus, severe acute respiratory syndrome coronavirus, hepatitis viruses and enteroviruses (11–15). As for hepatitis viruses, glycyrrhizin has been used to treat liver diseases, including chronic hepatitis B and C (10). Although glycyrrhizin decreases serum alanine aminotransferase concentrations in HCV-infected patients, it does not significantly reduce amounts of HCV RNA (16, 17). It has been reported that a glycyrrhizin-containing preparation reduces hepatic steatosis in transgenic mice expressing the full-length HCV polyprotein (18). Recently, anti-HCV activity of glycyrrhizin *in vitro* was reported (19, 20). However, clear evidence for it still appears to be lacking.

In this study, we used an HCV cell culture system to examine a methanol extract and a chloroform subfraction of *G. uralensis* and certain isolated compounds, as well as commercially available purified compounds, such as glycyrrhizin and glycyrrhetinic acid, for their anti-HCV activity. We report here that glycycoumarin, glycyrin, glycyrol and liquiritigenin isolated from *G. uralensis* showed anti-HCV activity whereas glycyrrhizin showed only a marginal anti-HCV activity. We also found that some other constituents of *G. uralensis* or of *G. inflata* and *G. glabra*, such as isoliquiritigenin, licochalcone A and glabridin, showed anti-HCV activity.

MATERIALS AND METHODS

Cells and viruses

Huh7.5 cells and the plasmid pFL-J6/JFH1 (21) were kindly provided by Dr. C. M. Rice (Rockefeller University, New York, NY, USA). Huh7.5 cells were cultured in Dulbecco's modified Eagle's medium supplemented with FBS (Biowest, Nuaillé, France), non-essential amino acids (Invitrogen, Carlsbad, CA, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen) at 37 °C in a 5% CO_2 incubator. A cell culture-adapted strain of HCV genotype 2a (J6/JFH1-P47) was prepared as described previously (22) and used in this study at an MOI of 2.0.

Extraction, sub-fractionation and purification of *G. uralensis* roots

G. uralensis roots were purchased from Tochimoto Tenkaido (Osaka, Japan). A methanol extract of G. uralensis roots was prepared and subjected to purification procedures, as described previously (23–26). In brief, *G. uralensis* roots were dried at room temperature and pulverized. They were then extracted with methanol at 50 °C for 6 hr. The extracts were filtered and the filtrates concentrated by using an evaporator at temperatures not exceeding 40 °C. The residues obtained were re-suspended in water and successively partitioned between chloroform and *n*-butanol. The chloroform extract was subjected to recycling preparative 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: Condition A) to afford 10 fractions (Fr.1 to Fr.10). Fr.7 was subjected to HPLC

separation (solvent system, acetonitrile–water; column, Imtakt Unison UK-C18C (Kyoto, Japan), 4.6 mm ID × 250 mm; flow rate, 2.0 mL/min; detection, UV 254 nm) to give 12 fractions (Fr.7–1 to 7–12) and glycyrin (2.5 mg; Fr.7–9). Fr.7–6 was purified by recycling HPLC (Condition A) to afford glycycoumarin (0.7 mg). Fr.8- to Fr.10 were combined and then re-chromatographed by HPLC (solvent system, acetonitrile–water; column, Imtakt Unison UK-C18C, 4.6 mm ID × 250 mm; flow rate, 2.0 mL/min; detection, UV 254 nm) to give 15 fractions (Fr.8–1 to Fr.8–15). Fr.8–3 was subjected to recycling HPLC (Condition A) to give liquiritigenin (1.2 mg). Fr.8–9 was purified by recycling HPLC (Condition A) to afford glycyrol (1.1 mg). The ^{1}H- and ^{13}C-NMR spectra were measured with a Jeol ECA 500 spectrometer (500 MHz; Tokyo, Japan). HPLC was performed on a JASCOLC-2000 plus system (Tokyo, Japan).

Chemicals

Glycyrrhizic acid (cat. no. 074-03481), glycyrrhizic acid mono-ammonium salt *n*-hydrate (cat. no. 075-02171), glycyrrhetinic acid (cat. no. 072-02181) and glabridin (cat. no. 070-04821) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Liquiritin (cat. no. L8045), liquiritigenin (cat. no. 78825) and licochalcone A (cat. no. 68783) were purchased from Sigma–Aldrich (Tokyo, Japan) and isoliquiritigenin (cat. no. I0822) from Tokyo Chemical Industry (Tokyo, Japan). Licorice-saponins G2 (cat. no. P2502) and H2 (cat. no. P2503), and glycyrrhetic acid 3-*O*-glucuronide (cat. no. NH080502) were purchased from Funakoshi (Tokyo, Japan). Glycycoumarin, glycyrol, glycyrin and liquiritigenin were isolated from *G. uralensis* extracts in this study, as described above.

Analysis of anti-HCV activity of plant extracts and purified compounds

Test samples were weighed and dissolved in DMSO to obtain stock solutions at 10 or 30 mg/mL. The stock solutions were stored at −20 °C until used. Huh7.5 cells were seeded in 24-well plates (1.6×10^5 cells/well). HCV was mixed with serial dilutions of the test samples (100, 30, 10, 3 and 1 μg/mL) and inoculated into the cells. After 2 hr, the cells were washed with medium to remove residual virus and further incubated in medium containing the same concentrations of the samples as those during virus inoculation. In time-of-addition experiments, treatment with the samples was performed only during or after virus inoculation in order to assess the mode of action of the samples examined. Culture supernatants were collected 1 and 2 days post-infection and titrated for virus infectivity, as described below. Virus and cells treated with medium containing 0.1% DMSO served as controls. Percent inhibition of the virus infectivity for each dilution of the samples was calculated by comparison with mock-treated controls and IC_{50} determined.

Virus titration

Virus samples were diluted serially 10-fold in complete medium and inoculated onto Huh7.5 cells seeded on glass coverslips in a 24-well plate. After virus adsorption for 2 hr, the cells were washed with medium to remove residual virus and cultured for 24 hr. The virus-infected cells were stained with an indirect immunofluorescence method as reported previously (27). In brief, the virus-infected cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After being washed three times with PBS, the cells were incubated with HCV-infected patient's serum for 1 hr, followed by incubation with FITC-conjugated goat anti-human IgG (MBL, Nagoya, Japan). The cells were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 5 min and HCV-infected cells were counted under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Cytotoxicity assay

The cytotoxicity of the samples was assessed by WST-1 assay as described previously with a slight modification (27). In brief, Huh7.5 cells in 96-well plates were treated with serial dilutions of the samples or 0.1% DMSO as a control for 48 hr. At the end of the treatment, 10 μL of WST-1 reagent (Roche, Mannheim, Germany) was added to each well and the cells cultured for 1 hr. 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 and 630 nm. Percent cell viability compared to the control was calculated for each dilution of the samples and CC_{50} were determined.

Immunoblotting

Cells were lysed with an SDS sample buffer, after which equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), as described previously (28, 29). The membranes were 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).

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as described previously (28, 29). In brief, total RNA was extracted from the cells using a ReliaPrep RNA cell miniprep system (Promega, Madison, WI, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using a GoScript Reverse Transcription system (Promega) with random primers and subjected to real-time quantitative 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, USA). The HCV-specific primers to amplify an NS5A region of the viral genome were 5′-AGACGTATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGCGACGGTGCTGATAG-3′ (antisense). Human GAPDH gene expression was measured using primers 5′-GCCATCAATGACCCCTTCATT-3′ (sense) and 5′-TCTCGCTCCTGGAAGATGG-3′, which served as an internal control.

RESULTS

Anti-HCV activity of a methanol extract and sub-fractions of *G. uralensis* roots

First, we examined a crude methanol extract of *G. uralensis* roots for anti-HCV activity using the HCV J6/JFH1-P47 strain and Huh7.5 cells. The IC_{50} and CC_{50} values of the crude methanol extract were 20.0 and 300 μg/mL, respectively, the selectivity indexes (SI: CC_{50}/IC_{50}) being 15 (Table 1). We further partitioned the methanol extract using different solvents and found that the anti-HCV activity was concentrated into a chloroform partition, whose IC_{50} and CC_{50} were 8.0 and 93 μg/mL, respectively, the SI being 11.6.

Table 1. Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of extracts of *G. uralensis* roots

Sample	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI
Methanol extract	20.0	300	15.0
Chloroform partition	8.0	93	11.6
n-butanol partition	17.5	250	14.3
Water partition	95.0	>500	>5.3

Table 2. Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of recycling HPLC fractions of chloroform partition of *G. uralensis* roots

Sample	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI
Fraction 1	>30	>30	na
Fraction 2	>30	>30	na
Fraction 3	>30	>30	na
Fraction 4	>30	>30	na
Fraction 5	20	>30	>1.5
Fraction 6	2.9	65	22
Fraction 7	3.6	40	11
Fraction 8	2.9	40	14
Fraction 9	2.9	>70	>24
Fraction 10	4.9	>70	>14

na, not applicable.

Next, we sub-fractionated the chloroform partition by using recycling HPLC and examined the sub-fractions for anti-HCV activity. We found significant anti-HCV activity with fractions 6–10, IC_{50} ranging between 2.9 and 4.9 μg/mL (Table 2). We further tried to purify a major component(s) in fractions 6–10 by using recycling HPLC: we identified glycycoumarin (30), glycyrin (31), glycyrol (32) and liquiritigenin (33) by NMR spectrum analysis (data not shown).

Anti-HCV activity of *G. uralensis*-derived purified compounds and related chemical compounds obtained from other *Glycyrrhiza* species

We then examined the purified compounds obtained from the *G. uralensis* extracts (glycycoumarin, glycyrin, glycyrol and liquiritigenin) and other *Glycyrrhiza*-derived chemical compounds, such as glycyrrhizin and its derivatives. We found that glycyrrhizin (glycyrrhizic acid) and its monoammonium salt (glycyrrhizic acid monoammonium), the latter being more water-soluble than the former, possessed only marginal anti-HCV activity, IC_{50} values being 180 and 320 μg/mL, respectively (Table 3). The aglycone of glycyrrhizin, glycyrrhetinic acid, showed more significant anti-HCV activity than did glycyrrhizin, its IC_{50} being 40 μg/mL. It should be noted that their anti-HCV activities were weaker than those of the recycling HPLC fractions 6–10 of the *G. uralensis* extract. The monoglucuronide form of glycyrrhizin, glycyrrhetic acid 3-*O*-glucuronide, did not show significant anti-HCV activity at the concentration tested. These results suggest that another compound(s) in fractions 6–10 was responsible for the anti-HCV activity. Two other saponins tested in this study, licorice-saponins G2 and H2, did not show detectable anti-HCV activity. On the other hand, coumarins, such as

Table 3. Anti-HCV activity (IC_{50}), cytotoxicity (CC_{50}) and selectivity index (SI) of *G. uralensis*- and other *Glycyrrhiza* spp-derived compounds

Sample	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI
Triterpenoids			
Glycyrrhizin	180	560	3.1
Glycyrrhizic acid monoammonium	320	690	2.2
Glycyrrhetic acid 3-O-glucuronide	>30‡	>30	na
Glycyrrhetinic acid	40.0	77.4	1.9
Licorice-saponin G2	>30‡	>30	na
Licorice-saponin H2	>30‡	>30	na
Coumarins			
Glycycoumarin (isolated)	8.8	69.0	7.8
Glycyrin (isolated)	7.2	25.0	3.5
Glycyrol (isolated)	4.6	35.5	7.7
Flavonoids			
Flavanones			
Liquiritin	75.0	570	7.6
Liquiritigenin	16.4	125	7.6
Chalcones			
Isoliquiritigenin	3.7	11.0	3.0
Licochalcone A†	2.5	20.0	8.0
Isoflavan			
Glabridin†	6.2	22.7	3.7

†Not detected in *G. uralensis* (34). ‡No detectable HCV inhibition at 30 μg/mL. na, not applicable.

glycycoumarin, glycyrin and glycyrol, which were isolated in this study (purity >90%) from the *G. uralensis* extracts, showed potent anti-HCV activity, IC_{50} being 4.6–8.8 μg/mL. Liquiritigenin, which was also isolated from the *G. uralensis* extracts, showed weaker anti-HCV activity than the coumarins. Also, isoliquiritigen, licochalcone A and glabridin exhibited potent anti-HCV activity, their IC_{50} being 3.7, 2.5 and 6.2 μg/mL, respectively. It should be noted that licochalcone A and glabridin have been reported to be absent in *G. uralensis* but present in *G. inflata* and *G. glabra* (34).

Mode-of-action of *G. uralensis*-derived isolated compounds and related compounds obtained from other *Glycyrrhiza* species

To determine whether the anti-HCV effects of the compounds of *Glycyrrhiza* species are exerted on the entry or post-entry stage, we performed the following time-of-addition experiments.

1. HCV was mixed with a test compound and the mixture inoculated into the cells. After virus adsorption for 2 hr, the residual virus and test sample were removed and the cells re-fed with fresh medium without the test sample for 46 hr. This experiment examines the antiviral effect at the entry step.
2. HCV was inoculated into the cells in the absence of a test sample. After virus adsorption for 2 hr, the residual virus was removed and the cells treated with the test samples for 46 hr. This experiment examines the antiviral effect at the post-entry step.
3. As a positive control, HCV mixed with the test sample was inoculated into the cells. After virus adsorption for 2 hr, the residual virus and test sample were removed and the cells treated with the test samples for 46 hr.

Table 4. Mode-of-action analysis of anti-HCV activities of *G. uralensis*- and other *Glycyrrhiza* spp.-derived compounds

Compound	Conc. (mg/mL)†	Anti-HCV activity (% inhibition) During‡	After‡	During & after‡
Chloroform partition	30	28.6	100	100
Glycycoumarin	20	16.7	100	100
Glycyrin	15	18.4	98.3	99.6
Glycyrol	10	21.3	100	100
Liquiritigenin	30	15.5	90.0	87.2
Isoliquiritigenin	8	14.1	91.0	82.5
Licochalcone A	5	0	94.4	93.8
Glabridin	12	0	91.0	93.8

†The concentrations of each compound used were 2 × IC_{50}. ‡Treatment with the compound was administered only during, only after or both during and after virus inoculation.

We found that all the *Glycyrrhiza* species-derived compounds as well as the chloroform partition of the *G. uralensis* extract exerted their antiviral effects after virus inoculation (Table 4). These results suggest that all the anti-HCV compounds of *Glycyrrhiza* species tested in this study, namely, glycycoumarin, glycyrin, glycyrol, liquiritigenin, isoliquiritigenin, licochalcone A and glabridin, as well as the chloroform extract, act primarily at the post-entry step.

Inhibition of HCV RNA replication and HCV protein synthesis by glycycoumarin, glycyrin, and glycyrol obtained from *G. uralensis*

To further confirm that *G. uralensis*-derived compounds exert their anti-HCV activities at the post-entry step, Huh7.5 cells were inoculated with HCV for 2 hr, followed by treatment with each of compounds for 1–2 days. The results obtained clearly demonstrated that glycycoumarin, glycyrin and glycyrol inhibit HCV RNA replication, resulting in decreased HCV protein

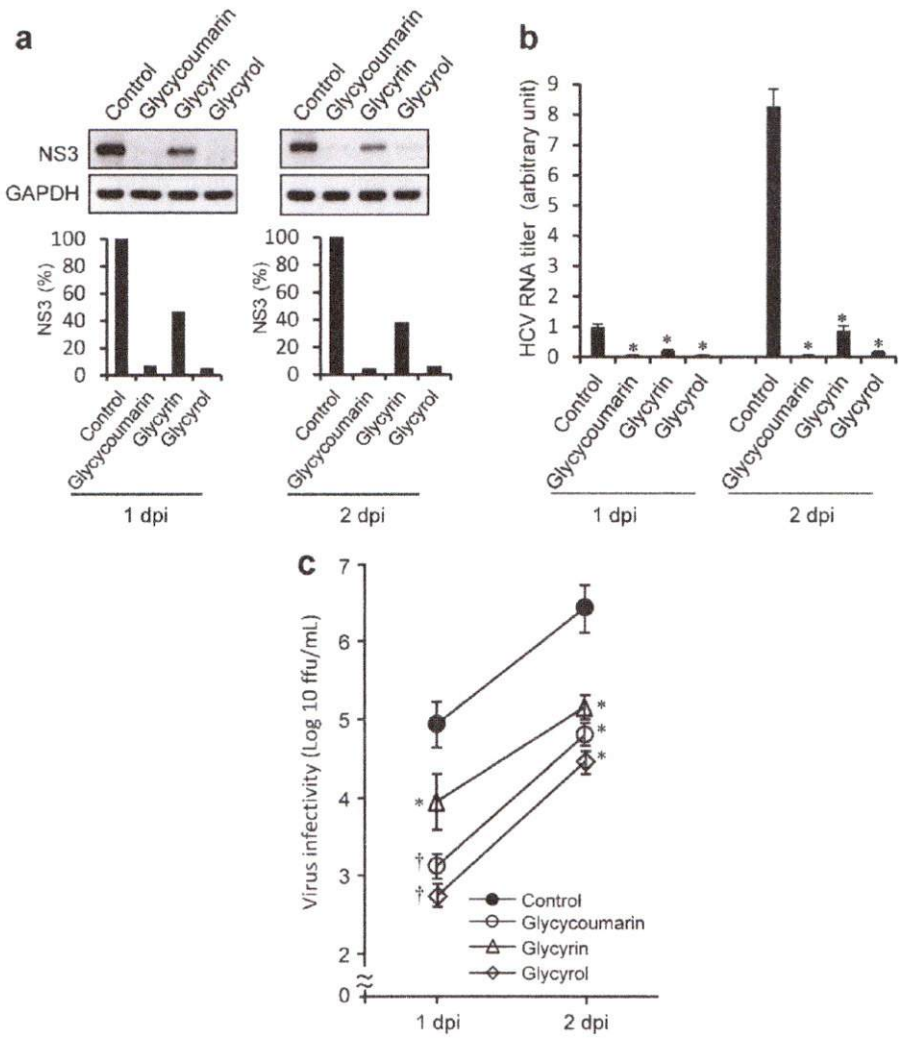

Fig. 1. Effects of glycycoumarin, glycyrin and glycyrol isolated from *G. uralensis* on HCV RNA replication and protein synthesis. (a) Huh 7.5 cells infected with HCV J6/JFH1 and treated with either glycycoumarin (20 μg/mL), glycyrin (15 μg/mL), glycyrol (10 μg/mL) or left untreated were subjected to western blot analysis using monoclonal antibody against the HCV NS3 protein at 1 and 2 days post-infection. GAPDH served as an internal control to verify equal amounts of sample loading. Signal intensities of NS3 were normalized to the corresponding GAPDH signal. (b) Amounts of HCV RNA in the cells described in (a) were measured by real-time quantitative RT-PCR analysis. These amounts were normalized to GAPDH mRNA expression. Data represent means ± SEM of data from two independent experiments. The value for the untreated control at 1 day post-infection is arbitrarily expressed as 1.0. $^{*}P < 0.001$, compared with the control. (c) Amounts of HCV infectious particles in the supernatants of the cultures described in (a) and (b) were determined: data for glycycoumarin, glycyrin, glycyrol and the untreated control are shown. Data represent means ± SEM of data from two independent experiments. $^{*}P < 0.05$; $^{\dagger}P < 0.01$, compared with the untreated control; dpi, days post infection.

synthesis as demonstrated by both real-time quantitative RT-PCR and immunoblotting analyses (Fig. 1a, b). We also confirmed that production of HCV infectious particles is inhibited by glycycoumarin, glycyrin and glycyrol at 1 and 2 days post-infection (Fig 1c).

DISCUSSION

Glycyrrhiza species possess a variety of bioactive compounds, such as: (i) triterpenoids, for example, glycyrrhizin, glycyrrhetic acid 3-*O*-glucuronide, glycyrrhetinic acid and various licorice-saponins; (ii) coumarins, for example, glycycoumarin, glycyrin and glycyrol; (iii) flavanones, for example, liquiritin and liquiritigenin; (iv) chalcones, for example, isoliquiritigenin and licochalcone; (v) isoflavans, for example, glabridin; (vi) stilbenoids, for example, dihydrostilbenes; and other miscellaneous compounds (11). In this connection, flavanones, chalcones and isoflavans are members of flavonoids.

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