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ORIGINAL PAPER

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Inhibitory effect of Yunnan traditional medicines on hepatitis C viral polymerase

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Abstract For the purpose of developing novel anti-hepatitis C virus (HCV) agents from natural resources, 93 Yunnan crude drugs were screened for their inhibitory effects on RNA-dependent RNA polymerase (RdRp) of HCV. Although 71 methanol extracts and 50 water extracts inhibited HCV-RdRp by more than 50% at a concentration of 50 μg/ml, the majority of them contained a high percentage of tannins. However, methanol extracts of *Plumbago zeylanica* (branch), *Maytenus fookerii* (leaf) and Huashidancha (Y61, branch and leaf), and water extracts of *Potentilla griffithii* (whole plant) and *Salvia yunnanensis* (underground part), having IC_{50} values of less than 10 μg/ml, showed less than 10% tannin content. In addition, from a methanol extract of *Tripterygium hypoglaucum* (root bark), demethylzeylasteral was isolated as a strongly inhibitory substance against HCV-RdRp.

Keywords *Tripterygium hypoglaucum* · Demethylzeylasteral · Hepatitis C virus · RNA-dependent RNA polymerase · Yunnan crude drugs

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Introduction

Hepatitis C virus (HCV) is an etiological agent of non-A, non-B hepatitis, which is a major public problem, infecting more than 170 million people worldwide [1, 2]. Infection with HCV is characterized by a high rate of chronicity, and patients infected with HCV are at high risk of developing liver cirrhosis and hepatocellular carcinoma [3–6]. In the absence of a prophylactic vaccine or highly specific antiviral agent, treatment options for individuals with chronic infection are limited [7]. The current approved therapies with interferon are only partially effective and treatment is accompanied by undesirable side effects [8]. Furthermore, many of those who begin therapy with the current standard interferon alpha and ribavirin do not achieve viral clearance. In some cases, the treatment failure can be traced to drug side effects and/or patient non-adherence to a long-term course of therapy [9]. New treatment agents are required that are more efficacious and safe for patients.

HCV is a positive-stranded RNA virus with the genome size of approximately 9.4 kb, containing an ORF, encoding a 3000 amino acid polyprotein. This polyprotein is the precursor to all viral proteins processed by viral and cellular proteases into at least nine different viral polypeptides [10, 11]. The section of the polypeptide chain where the key enzyme, NS5B protein, acts as a viral specific RNA-dependent RNA polymerase (RdRp) in addition to being the catalytic core of the viral replicase [8]. Viral specific enzymes are thought to be the most suitable target for HCV. Although all HCV enzymes are equally appropriate for development of new therapy, the NS3-4A serine protease and the NS5B RNA polymerase have emerged as the most popular targets. In early clinical trails, steps were made toward developing drugs that target the viral enzyme, or gemone, as well as novel immunomodulatory molecules, with particular emphasis on agents with demonstrated antiviral activity. However, clinical developments of some inhibitors were stopped

because of the observation of adverse effects in laboratory animals [12].

In the course of our studies on development of HCV-RdRp inhibitors from naturally occurring products, we focused on traditional medicinal plants. The Yunnan crude drugs studied were characterized by their use by minority people in China as folk medicines. In this paper, we report the screening of methanol and water extracts, prepared from the Yunnan crude drugs, for their inhibitory activity against the viral specific enzyme HCV-RdRp, with activity represented as a 50% inhibitory concentration (IC_{50}). In addition, we report anti-HCV-RdRp substances isolated from the root bark of *Tripterygium hypoglaucum*.

Materials and methods

Plant materials

Medicinal plants (crude drugs) used in this study were collected in Yunnan Province of China in 2000, during the Japan Society for the Promotion of Science (JSPS) Project of Overseas Survey on Ethnical Medicines [12–15]. The root barks of *T. hypoglaucum* were obtained from Kunming, Yunnan Province, China, and the botanical source was identified by K.K. These specimens are deposited in the Herbarium of Materia Medica of University of Toyama (TMPW no. 24248, 24249).

Preparation of extracts

The crude drugs (5 or 10 g) were pulverized and extracted separately with water and methanol. The solutions were evaporated in vacuo to give a residue that was then dissolved in DMSO and water and used in the following assays.

RdRp assay

Recombinant HCV-RdRp was prepared as reported previously [13, 17]. For the assay of HCV-RdRp activity, a reaction mixture (20 µl) containing 20 m*M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 m*M* EDTA (pH 8.0), 1 µg/ml Poly(rA), 1 µg/ml Oligo(U) as a template primer, 10 μM UTP, 7.4×10^3 Bq [α-^{32}P] UTP, HCV-NS5B, and 1.0 µl of a test compound dissolved in DMSO/H_2O (1:1) (final DMSO concentration of 2.5%) was incubated at 25°C for 1 h. A blank reaction was carried out under the same conditions without adding the enzyme, and a control reaction was carried out under the conditions without adding a test compound. The mixture was poured into ice-water to terminate the reaction. The resulting mixture was applied to a Whatman DE81 ion exchange paper disc, which was washed batch-wise with 3 ml 0.5 *M* Na_2HPO_4 four times, then once with water, once with ethanol, and once with ether. The paper disc was dried and immersed in 3 ml scintillation fluid, and the radioactivity was counted on a scintillation counter. The percentage inhibition was calculated from the following equation:

$$\text{Inhibition}(\%) = [1 - (\text{dpm sample}/\text{dpm control})] \times 100$$

Adriamycin was used as a positive control.

Measurement of tannin content [18]

A 2-ml aliquot of 0.2 mg/ml methanol or water extract was mixed with 0.2 *M* phosphate buffer (pH 7.0, 1 ml) and 26 µg/ml methylene blue solution (2 ml). The mixture was left at room temperature for 30 min and centrifuged at 1500 *g* for 10 min. The absorbance of the supernatant was measured at 660 nm. The standard curve was obtained using a solution of tannic acid (Nacalai Tasque, Kyoto, Japan). The tannin content was calculated in terms of tannic acid.

Extraction and isolation

Procedure 1 The root bark of *T. hypoglaucum* (3.3 kg) was extracted with MeOH under reflux (5 l×3) for 3 h. The combined solutions were evaporated to dryness in vacuo to yield a MeOH extract. The MeOH extract (218.8 g) was suspended in water (500 ml) and extracted with EtOAc (1.5 l). The EtOAc phase was evaporated to dryness to yield an EtOAc extract (53.6 g), which was further partitioned between hexane and MeOH to yield the respective extracts in yield of 10.1 and 41.3 g. Linoleic acid (compound 1, 92.8 mg), palmitic acid (compound 2, 497 mg), oleanolic acid 3-*O*-acetate (compound 3, 16.3 mg), and β-sitosterol (compound 4, 110 mg) were obtained from a hexane extract by repeated column chromatography (CC) on silica gel, eluted with hexane-EtOAc (10:0 → 0:10). The H_2O phase was concentrated and applied to a Diaion HP-20 column and the column was eluted stepwise with H_2O and MeOH. The respective eluates were evaporated to dryness in vacuo to yield two fractions (25.1 g and 28.7 g, respectively). Demethylzeylasteral (compound 5) [18, 19] was obtained from the MeOH-eluted fraction by repeated CC on silica gel, eluted with $CHCl_3$-MeOH (99:1 → 100:1) and crystallized from Me_2CO to yield a pure compound (compound 5, 10 mg).

Procedure 2 The root bark of *T. hypoglaucum* (5.5 kg) was extracted with Me_2CO at room temperature (5 l×3). The Me_2CO extract (305 g) was concentrated and applied to an MCI CHP 20P column and the column was eluted stepwise with 30% MeOH-H_2O, 60% MeOH-H_2O, 90% MeOH-H_2O, and 100% MeOH. The respective eluates were evaporated to dryness in vacuo to yield four fractions (94.9 g, 79 g, 29.4 g and 3.98 g, respectively). Demethylzeylasteral (compound 5) and

celastrol [19, 20] (compound 6, 467 mg) were obtained from the MeOH-eluted fraction by repeated CC on Sephadex LH-20, eluted with EtOH-$CHCl_3$ (7:3). Demethylzeylasteral (compound 5) was crystallized from Me_2CO in a yield of 155 mg. The chemical structures of these compounds were determined by their IR spectra (JASCO FT/IR-230 infrared spectrometer), UV spectra (Shimadzu UV-2200 UV-VIS recording spectrometer), CD spectra (JASCO J-805 spectropolarimeter), ^1H and ^{13}C NMR spectra (Varian UNITY plus 500 or Varian Gemini 300 NMR spectrometer), and optical rotation data (JASCO DIP-360 automatic polarimeter).

Characterization of isolated compounds and their derivatives

Demethylzeylasteral (compound 5) Yellow solid. ^1H NMR (CD_3OD, 500 MHz): δ 0.75 (3H, s, 20β-CH_3), 1.13 (3H, s, 13-CH_3), 1.18 (3H, s, 17-CH_3), 1.36 (3H, s, 14-CH_3), 1.57 (3H, s, 9-CH_3), 6.30 (1H, s, 1-H), 7.26 (1H, s, 7-H), 10.84 (1H, s, CHO). ^{13}C-NMR (CD_3OD, 125 MHz) δ 19.3 (C-27), 21.2 (C-26), 29.9 (C-15), 30.7 (C-12), 30.8 (C-21), 31.6 (C-17), 31.8 (C-19), 32.0 (C-28), 33.2 (C-30), 34.7 (C-11), 35.9 (C-22), 36.8 (C-25), 37.5 (C-16), 40.6 (C-13), 41.2 (C-20), 41.8 (C-9), 45.6 (C-18), 46.3 (C-14), 117.7 (C-1), 117.8 (C-4), 123.1 (C-5), 125.6 (C-7), 151.5 (C-3), 152.4 (C-2 and C-10), 176.6 (C-8), 182.4 (C-29), 187.6 (C-6), and 200.5 (C-23).

Celastrol (compound 6) Orange powder. ^1H NMR (CD_3OD, 500 MHz): δ 0.56 (3H, s, 20-CH_3), 1.08, 1.23, 1.26 (3×3H, s, 17-CH_3, 14-CH_3, 13-CH_3), 1.42 (3H, s, 9-CH_3), 2.20 (3H, s, 4-CH_3), 6.32 (1H, d, J = 7.2 Hz, H-7), 6.49 (1H, br s, H-1), 7.07 (1H, br d, J = 7.2 Hz, H-6). ^{13}C NMR (CD_3OD, 125 MHz): δ 10.4 (C-23), 18.7 (C-27), 21.4 (C-26), 28.6 (C-15), 29.2 (C-21), 29.4 (C-12), 30.6 (C-17), 31.0 (C-19), 31.4 (C-28), 32.4 (C-30), 33.7 (C-11), 34.4 (C-22), 36.3 (C-16), 38.3 (C-25), 39.2 (C-13), 39.9 (C-20), 43.0 (C-9), 44.2 (C-18), 45.3 (C-14), 118.2 (C-7), 120.2 (C-4), 120.4 (C-1), 127.4 (C-5), 135.5 (C-6), 146.9 (C-3), 165.0 (C-10), 172.6 (C-8), 178.3 (C-2), and 182.8 (C-29).

2,3-Diacetoxy-6,23-dioxo-24-nor-1,3,5(10),7-friedelatetraen-29-oic acid (compound 5a) Demethylzeylasteral (10 mg) was treated with Ac_2O (3 ml) and pyridine (3 ml) for 1 h at room temperature. A few pieces of ice were added and the mixture was stirred for 10 min and extracted with $CHCl_3$ (3 ml). The $CHCl_3$ layer was washed successively with 1 *N* HCl and water saturated with NaCl. The $CHCl_3$ layer was dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated in vacuo and chromatographed on Sephadex LH-20 eluted with 100% $CHCl_3$ to yield 2,3-diacetoxy-6,23-dioxo-24-nor-1,3,5(10),7-friedelatetraen-29-oic acid (compound 5a, 9.9 mg): yellow amorphous powder; ^1H NMR ($CDCl_3$, 500 MHz): δ 0.61 (3H, s, 20β-CH_3), 1.05 (3H, s, 13-CH_3), 1.08 (3H, s, 17-CH_3), 1.27 (3H, s, 14-CH_3), 1.52 (3H, s, 9-CH_3), 2.31 (3H, s, C$\underline{H}_3$CO-), 2.36 (1H, s, C$\underline{H}_3$CO-), 6.34 (1H, s, H-7), 7.43 (1H, s, H-1), 10.93 (1$\underline{H}$, s, CHO). ^{13}C NMR (CD_3OD, 75 MHz): δ 18.7, 20.4, 20.5 ($\underline{C}H_3$CO-), 20.6 ($\underline{C}H_3$CO-), 28.5, 29.3, 29.4, 30.3, 30.4, 31.5, 32.4, 33.5, 34.5, 36.1, 36.8, 39.1, 40.1, 40.3, 44.0, 45.1, 117.8, 124.8 (C-4), 125.6, 128.1, 142.7, 147.9, 153.5, 167.4 ($CH_3\underline{C}O$-), 167.8 ($CH_3\underline{C}O$-), 174.6, 183.6 (C-29), 185.7 (C-6), 199.1 (C-23). IR ($CHCl_3$) cm^{-1}: 3029, 2943, 1731, 1645, 1457, and 1372. UV λ_{max} (MeOH) nm (log ε): 284 (3.80), sh (3.49), 370 (3.23). FAB-MS m/z: 566 $(M+H)^+$, 540, 523, 465, 229, 203, 149, 95, and 55. HRFAB-MS m/z: 565.2797 (calculated for $C_{33}H_{41}O_8$: 565.2801). $[\alpha]_D^{25}$–16.0° (c, 0.01, MeOH).

2,3,23-Trihydroxy-24-nor-6-oxo-1,3,5(10),7-friedelatetraen-29-oic acid (Compound 5b) Demethylzeylasteral (10 mg) was dissolved in MeOH (3 ml) and treated with $NaBH_4$ (5 mg) for 1 h at room temperature. The reaction mixture was worked up as usual to yield 2,3,23-trihydroxy-24-nor-6-oxo-1,3,5(10),7-friedelatetraen-29-oic acid (compound 5b, 3 mg): yellow amorphous powder; ^1H NMR (CD_3OD, 300 MHz): δ 0.75 (3H, s, 20β-CH_3), 1.13 (3H, s, 13-CH_3), 1.18 (3H, s, 17-CH_3), 1.35 (3H, s, 14-CH_3), 1.55 (3H, s, 9-CH_3), 4.80, 5.14 (each 1H, ABq, J = 12.2 Hz, 23-H_2), 6.26 (1H, s, H-1), 6.97 (1H, s, H-7). ^{13}C NMR (CD_3OD, 75 MHz): δ 19.5 (C-27), 21.4 (C-26), 29.9 (C-15), 30.8 (C-12), 31.7 (C-17 and C-21), 31.9 (C-19), 32.1 (C-28), 33.2 (C-30), 35.1 (C-11), 36.0 (C-22), 37.6 (C-25), 37.9 (C-16), 40.6 (C-13), 41.3 (C-20), 41.8 (C-9), 45.6 (C-18), 46.1 (C-14), 58.0 (C-23) 111.2 (C-1), 122.5 (C-4), 125.0 (C-5), 127.2 (C-7), 143.9 (C-3), 151.9 (C-10), 152.8 (C-2), 175.9 (C-8), 182.3 (C-29), and 189.4 (C-6). IR (KBr) cm^{-1}: 3423, 2943, 1700, 1636, 1578, 1459, 1378, and 1320. UV λ_{max} (MeOH) nm (log ε): 262 (4.71), 305 (4.44), 370 (4.38). FAB-MS m/z: 484 $(M+H)^+$, 466, 215, 154, 136. HRFAB-MS m/z: 483.2752 (calculated for $C_{29}H_{39}O_6$: 483.2746). $[\alpha]_D^{25}$–330° (c, 0.002, MeOH).

Compound 5c Demethylzeylasteral (5 mg) dissolved in $CHCl_3$ (1 ml) was stirred with Ag_2O (30 mg) and iodomethane (0.5 ml) for 2 h at room temperature. The reaction was carried out in accordance with available literature [19] to yield compound 5c, dimethylzeylasteral, quantitatively: ^1H NMR (CD_3OD, 300 MHz): δ 0.61 (3H, s, 20β-CH_3), 1.14 (3H, s, 13-CH_3), 1.17 (3H, s, 17-CH_3), 1.39 (3H, s, 14-CH_3), 1.64 (3H, s, 9-CH_3), 3.55 (3H, s, CO_2CH_3), 3.79, 4.01 (2×3H, s, 2×OCH_3), 6.30 (1H, s, 1-H), 7.27 (1H, s, 7-H), 10.31 (1H, s, CHO).

Compound 6a Celastrol (16 mg) was dissolved in MeOH (3 ml) and treated with $NaBH_4$ (5 mg) for 1 h at room temperature. The reaction was carried out in accordance with available literature [21] to yield triptohypol C (compound 6a, 8.7 mg): ^1H NMR (C_5D_5N, 500 MHz): δ 1.08 (3H, s, 20β-CH_3), 1.14 (3H, s, 13-CH_3), 1.24 (3H, s, 17-CH_3), 1.37 (3H, s, 14-CH_3), 1.43

(3H, s, 9-CH_3), 2.36 (3H, s, 4-CH_3), 3.08 (1H, d, $J = 20.7$ Hz, Hα-6), 3.30 (1H, dd, $J = 20.7$, 5.6 Hz, Hβ-6), 5.83 (1H, d, $J = 5.6$ Hz, H-7), 7.13 (1H, s, H-1).

Results

Screening of crude drug extracts for HCV-RdRp inhibitory activity

About 93 Yunnan traditional medicines were screened for inhibitory effect on RdRp of HCV (Table 1). Inhibitory activity was shown by 35 methanol extracts and 50 water extracts at a concentration of 50 μg/ml (data not shown). Inhibitory activity was evaluated based on the respective IC_{50} values of the extracts (Tables 2 and 3). The IC_{50} values ranged from 0.6 to 40.9 μg/ml. The following 13 extracts showed IC_{50} values less than 10 μg/ml: methanol extracts of *T. hypoglaucum* (Y44), *Polygonum cymosum* (Y53), *Ceratostigma willmottianum* (Y54), *Dioscorea cirrhosa* (Y56), *Maytenus fookerii* (Y58), and Huashidancha (Y61), and water extracts of *P. cymosum* (Y53), *C. willmottianum* (Y54), *D. cirrhosa* (Y56), *M. fookerii* (Y58), *Coptis teetoides* (Y73), and *Salvia yunnanensis* (Y90). Since, most of the crude drugs have been reported to contain various tannins that bind to proteins non-specifically [13], the tannin contents of the 85 inhibitory extracts were measured using a methylene blue method (Tables 2 and 3) [18]. Some of the extracts showed high tannin contents, approximately 10% or higher. For verifying whether their inhibitory effect was due to nonspecific binding of tannins and proteins including the enzyme or not, an enzyme reaction was carried out in the presence of bovine serum albumin (BSA). Most of the extracts showed less inhibitory activity in the presence of BSA (Tables 2 and 3). However, the inhibitory activities of methanol extracts of *Thalictrum glandulosissimum* (Y5), Shihuacai (Y9), *Cladonia fallax* (Y19), *Begonia evansiana* (Y27), *T. hypoglaucum* (Y44), *Plumbago zeylanica* (Y45), *C. willmottianum* (Y54), *C. teetoides* (Y73), and *Valeriana jatamansi* (Y78), and water extracts of *T. hypoglaucum* (Y38), *T. hypoglaucum* (Y44), *P. zeylanica* (Y45), *Piper hancei* (Y50), *Kadsura longipedunculata* (Y52), *C. willmottianum* (Y54), *M. fookerii* (Y58), Huashidancha (Y61), *Potentilla griffithii* (Y63), *Phellodendron chinense* (Y64), and *Moghania philippinensis* (Y86) were not significantly reduced in the presence of BSA.

Isolation of HCV-RdRp inhibitory compounds from *T. hypoglaucum*, and preparation of their derivatives

Extracts of *T. hypoglaucum* showed relatively high HCV-RdRp inhibitory activity (Table 2) and its inhibitory activity was maintained after eliminating the inhibitory effect of tannins by the addition of BSA to the enzyme reaction mixture. The percentage inhibition of

Table 1 List of Yunnan medical plants

Sample no.	Botanical name	Part used
Y1	*Thalictrum glandulosissimun* (Fin. et Gagn.) W. T. Wang et S. H. Wang	Underground part
Y2	*Phallus impudicus* Linn. ex Pers.	Fungus
Y3	*Taxus mairei* (Lemee et Levl.) S. Y. Hu or *Taxus Wallichinana* Zucc.	Bark
Y4	*Rhodiola sacra* (Prain ex Hamet) S. H. Fu	Underground part
Y5	*T. glandulosissimum* (Fin. et Gagn.) W. T. Wang et S. H. Wang	Underground part
Y6	*Acorus calamus* L.	Rhizome
Y7	*Sausurea namikawae* Kitam.	Whole plant
Y8	*Malus yunnanensis* (Franch.) Schneid. or *M. prunifolia* (Willd.) Borkh.	Fruit
Y9	Botanical name unknown	Whole plant
Y10	*Lentinus edodes* (Berk.) Sing.	Fungus
Y11	*Eucheuma gelatinae* (Esp.) J. Ag.	Alga
Y12	*Strobilomyces floccopus* (Vahl. ex Fr.) Karst. or *S. grevillei* (Kl.) Sing. or *S. luteus* (Linn. ex Fr.) Gray	Fungus
Y13	*Cynanchum otophyllum* Schneid.	Root
Y14	*Lycopus lucidus* Turcz.	Rhizome
Y15	*Lobaria retigera* Trevis.	Fungus
Y16	*Auricularia auricula* (L. ex Hook.) Underw.	Fungus
Y17	*Codonopsis subscaposa* Kom.	Root
Y18	*Panax notoginseng* (Burkill) F. H. Chen	Flower
Y19	*C. fallax* Abbayes	Whole plant
Y20	*Sargentodoxa cuneata* (Oliv.) Rehd. ex Wils.	Wood
Y21	*Ligusticum delavayi* Franch.	Root, rhizome
Y22	*Rhodobryum giganteum* (Schwaegr.) Par.	Whole plant
Y23	*Saussurea laniceps* Hand.-Mazz.	Whole plant
Y24	*Smilax ferox* Wall. ex Kunth	Rhizome
Y25	*Thamnolia vermicularis* (Sw.) Ach. ex Schaer.	Whole plant
Y26	*Aucklandia lappa* Dence.	Root
Y27	*B. evansiana* Andr.	Wood
Y28	*Paris polyphylla* Smith var. *yunnanenis* (Franch.) Hand.-Mazz.	Rhizome
Y29	*Verbena officinalis* L.	Whole plant
Y30	*Brandisia cauliflora* Tsoong et Lu	Branch, leaf
Y31	*Akebia trifoliata* (Thunb.) Koidz. var. *australis* (Diels) Rehd.	Branch
Y32	*Schisandra henryi* Clarke var. *marginalis* A. C. Smith or *S. henryi* Clarke var. *yunnanenis* A. C. Smith	Branch
Y33	*Millettia bonatiana* Pamp.	Branch
Y34	*Anemone rivularis* Buch.-Ham.	Branch
Y35	*Tupistra chinensis* Bak.	Rhizome
Y36	*Sarcococca ruscifolia* Stapf	Branch, leaf
Y37	*Passiflora cupiformis* Mast.	Stem
Y38	*T. hypoglaucum* Planch.	Stem
Y39	*Artemisia annua* L.	Whole plant
Y40	*Dysosma versipellis* (Hance) M. Cheng	Rhizome
Y41	*Periploca calophylla* (Wight) Falc.	Branch, leaf
Y42	*Tripterygium forrestii* Loesen. or *T. hypoglaucum* (Levl.) Hutch.	Branch
Y43	*Toddalia asiatica* (L.) Lam.	Branch

Table 1 (Contd.)

Sample no.	Botanical name	Part used
Y44	*T. hypoglaucum* (Levl.) Hutch.	Root bark
Y45	*P. zeylanica* L.	Branch
Y46	Botanical name unknown	Root
Y47	*Paederia scandens* (Lour.) Merr.	Stem
Y48	*B. cauliflora* Tsoong et Lu	Branch, leaf
Y49	*Clematis armandii* Franch.	Heart wood
Y50	*P. hancei* Maxim.	Whole plant
Y51	*Rumex nepalensis* Spreng.	Rhizome, root
Y52	*K. longipedunculata* Finet et Gagn.	Branch
Y53	*P. cymosum* Trev.	Root
Y54	*C. willmottianum* Staph	Branch
Y55	*Silene asclepiadea* Franch. (= *Melandrium viscidulum* var. *szechuanense* (Williams) Hand.-Mazz.)	Root
Y56	*D. cirrhosa* Lour.	Root
Y57	*Veratrum taliense* Loesen. f. or *V. mengtzeanum* Loesen. f.	Root
Y58	*M. fookerii* Loes.	Leaf
Y59	*Rauvolfia yunnanensis* Tsiang	Root
Y60	*M. fookerii* Loes.	Branch
Y61	Botanical name unknown	Branch, leaf
Y62	*Hedyotis diffusa* Willd. or *H. tenelliflora* Bl. or *H. corymbosa* (L.) Lam.	Root
Y63	*P. griffithii* Hook. f. var. *velutina* Card.	Whole plant
Y64	*P. chinense* Schneid.	Branch, stem
Y65	*Iris tectorum* Maxim.	Branch
Y66	*Bergenia purpurascens* (Hook. f. et Thoms.) Engl.	Root
Y67	*Dioscorea nipponica* Makino	Rhizome
Y68	*Gastrodia elata* Bl.	Rhizome
Y69	*Panax japonicus* C. A. Meyer var. *major* (Burk.) C. Y. Wu et K. M. Feng	Rhizome, root
Y70	*Engleromyces goetzii* P. Henn	Fungus
Y71	*Panax japonicus* C. A. Meyer var. *major* (Burk.) C. Y. Wu et K. M. Feng	Rhizome, root
Y72	*Viscium coloratum* (Kom.) Nakai	Whole plant
Y73	*C. teetoides* C. Y. Cheng	Rhizome
Y74	*Typhonium trilobatum* (L.) Schott	Root
Y75	*A. calamus* L.	Rhizome
Y76	*Tinospora yunnanensis* S. Y. Hu	Root
Y77	*Scutellaria amoena* C. H. Wright	Root
Y78	*Valeriana jatamansi* Jones	Root
Y79	Botanical name unknown	Whole plant
Y80	*Tacca plantaginea* (Hance) Drench.	Root
Y81	*Menispermum dauricum* DC.	Root
Y82	*Heracleum scabridum* Franch.	Root
Y83	*Campsis grandiflora* (Thunb.) Loisel.	Stem
Y84	*Pueraria phaseoloides* (Roxb.) Benth.	Root
Y85	*Scutellaria barbata* D. Don	Whole plant
Y86	*M. philippinensis* (Merr. et Rolfe) Li	Root
Y87	*Eleutherine americana* Merr. et Heyne	Bulb
Y88	*Saussurea medusa* Maxim.	Whole plant
Y89	*Saussurea involucrata* Kar. et Kir.	Whole plant
Y90	*S. yunnanensis* C. H. Wright	Underground part
Y91	*Rheum tanguticum* Maxim. ex Balf.	Rhizome
Y92	*Crocus sativas* L.	Flower
Y93	*Swertia mussotii* Franch.	Whole plant

Table 2 Inhibitory activity of methanol extracts of Yunnan medical plants on HCV-RdRp. The inhibitory effects of the extracts were measured in the presence (+) or absence (−) of 1 mg/ml bovine serum albumin (*BSA*). The values are means ($n = 2$)

Sample no.	Inhibition of HCV RdRp (%) 50 μg/ml		IC_{50} (μg/ml)	Tannin content (%)
	BSA (−)	BSA (+)		
Y5	55.5	92.0	24.9	< 0.5
Y6	55.5	0	36.9	< 0.5
Y9	104.9	78.6	16.5	< 0.5
Y11	80.0	41.6	17.0	< 0.5
Y12	68.4	0	21.8	< 0.5
Y19	65.1	92.0	27.5	< 0.5
Y20	73.2	19.5	14.2	< 0.5
Y24	74.5	24.2	23.5	< 0.5
Y27	91.8	73.6	11.5	3.1
Y32	80.6	12.7	19.6	1.2
Y38	109.4	57.9	9.2	10.9
Y41	92.7	53.8	15.9	< 0.5
Y42	100.8	2.7	11.4	< 0.5
Y44	55.8	67.3	29.4	0.8
Y45	66.0	89.1	209	9.8
Y51	77.9	26.6	15.0	14.0
Y52	61.6	20.7	20.2	7.7
Y53	86.6	50.7	5.2	25.0
Y54	88.7	83.2	3.1	13.6
Y56	84.7	59.1	0.6	21.2
Y58	87.2	46.8	4.6	< 0.5
Y61	89.3	62.0	1.6	9.5
Y63	93.2	48.0	11.0	3.3
Y66	84.0	0	14.7	44.9
Y67	51.5	0	37.2	< 0.5
Y73	62.7	82.6	14.5	< 0.5
Y77	60.9	0	29.1	18.9
Y78	59.7	79.6	34.2	< 0.5
Y84	81.7	0	17.4	< 0.5
Y85	54.6	12.1	41.6	< 0.5
Y86	61.9	33.7	20.0	< 0.5
Y87	72.2	0	17.8	< 0.5
Y90	88.8	7.2	16.8	< 0.5
Y91	100.4	60	10.5	3.8
Y93	97.0	39.8	10.6	< 0.5

the methanol extract of *T. hypoglaucum* was 85.1% at 100 μg/ml against HCV-RdRp (data not shown). The substances inhibiting HCV-RdRp in the extract were further investigated by bioassay-guided fractionation. A MeOH-eluted fraction of the MeOH extract showed appreciable inhibitory activity, from which demethylzeylasteral (compound 5) was isolated as the most potent inhibitory substance (Table 4). Under conditions in which demethylzeylasteral (compound 5) possessed potent activity with an IC_{50} value of 7.4 μM, celastrol (compound 6) showed an IC_{50} value of 36.4 μM. On the other hand, compounds isolated from a hexane extract, including linoleic acid (compound 1), palmitic acid (compound 2), oleanolic acid 3-*O*-acetate (compound 3) and β-sitosterol (compound 4) did not show any significant HCV-RdRp inhibitory activity at concentrations of 50 and 100 μM.

In an attempt to correlate activities on a structural basis, several demethylzeylasteral derivatives were prepared and their HCV-RdRp inhibitory activities com-

Table 3 Inhibitory activity of water extracts of Yunnan medical plants on HCV-RdRp. The inhibitory effects of the extracts were measured in the presence (+) or absence (−) of 1 mg/ml bovine serum albumin (*BSA*). The values are means ($n=2$)

Sample no.	Inhibition of HCV-RdRp (%) 50 μg/ml		IC_{50} (μg/ml)	Tannin content (%)
	BSA (−)	BSA (+)		
Y1	52.3	5.0	37.8	< 0.5
Y5	63.7	11.9	29.5	< 0.5
Y7	57.9	0.1	28.8	< 0.5
Y8	35.7	9.0	35.7	< 0.5
Y9	16.5	0	16.5	< 0.5
Y11	32.3	6.9	32.3	< 0.5
Y12	22.8	0	22.8	< 0.5
Y15	70.9	0	21.7	< 0.5
Y20	73.9	55.5	20.1	13.4
Y22	57.9	0	19.0	< 0.5
Y23	55.8	0	35.0	< 0.5
Y25	87.2	16.3	30.6	< 0.5
Y26	59.9	0	40.7	< 0.5
Y27	110.4	53.9	11.5	8
Y29	84.8	0	40.0	< 0.5
Y30	58.9	0	40.9	< 0.5
Y31	85.8	0	19.4	< 0.5
Y32	63.2	4.5	30.4	< 0.5
Y34	80.2	49.2	14.0	3.1
Y38	87.1	69.9	12.0	< 0.5
Y39	70.5	5.9	16.0	22.9
Y42	70.7	49.4	23.9	12.3
Y44	67.2	58.1	32.0	1.3
Y45	92.6	92.3	12.6	33.6
Y50	87.8	71.2	13.6	3.8
Y51	79.5	49.1	18.3	9.5
Y52	80.2	70.8	14.4	19.6
Y53	72.3	52.3	7.7	19.5
Y54	81.0	66.5	6.0	26.5
Y56	70.4	49.1	9.5	13.2
Y58	79.4	72.0	8.9	27.9
Y60	81.2	34.6	13.7	11.3
Y61	82.7	82	11.3	1.9
Y63	95.8	88.7	8.7	< 0.5
Y64	57.4	72.5	36.2	32.1
Y66	58.1	24.6	28.9	< 0.5
Y73	85.8	52.0	8.0	40.0
Y75	64.9	18.2	31.0	< 0.5
Y77	67.2	11.5	24.9	5.4
Y78	74.1	5.7	19.7	< 0.5
Y79	67.7	24.0	27.0	< 0.5
Y81	70.4	57.2	26.0	< 0.5
Y82	59.9	17.1	30.6	< 0.5
Y84	85.6	28.0	16.5	< 0.5
Y85	86.2	7.0	20.7	1.5
Y86	91.2	75.3	10.7	12.7
Y87	85.9	60.8	16.8	< 0.5
Y90	95.2	22.5	7.4	1.9
Y91	92.6	61.4	6.9	19.4
Y93	60.0	44.6	31.9	< 0.5

pared (Table 4). Although demethylzeylasteral (compound 5) showed potent inhibitory activity, addition of acetyl groups to the parent compound resulted in a decrease in inhibitory activity (compound 5a). Similarly, reduction of an aldehyde group in compound 5 to a hydroxymethyl group (compound 5b) reduced the inhibitory activity. Furthermore, methylation of compound 5 (compound 5c) led to no inhibition against HCV-RdRp. Celastrol derivatives (compound 6a) showed relatively high inhibitory activity with an IC_{50} value of 24.6 μM.

Table 4 Inhibitory activity of compounds isolated from the root bark of *T. hypoglaucum* and their derivatives. Values are means ($n=2$)

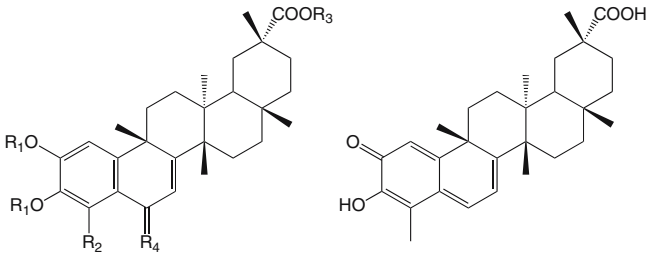
Compound	Inhibition (%) at 50 μM	IC_{50} (μM)
1	0	> 100
2	6.2	> 100
3	0	> 100
4	0	> 100
5	84.3	7.4
5a	62.5	38.1
5b	31.5	100
5c	1.3	> 100
6	63.3	36.4
6a	66.2	24.6

Discussion

Some crude drugs collected in Yunnan Province of China screened for their inhibitory activity against HCV-RdRp showed appreciable inhibition when evaluated in terms of their IC_{50} value, either in the presence or absence of BSA. These crude drug extracts seem to be potential candidates for development of HCV-RdRp-inhibitory substances.

A traditional Chinese prescription, Ninjinyoeito (TJ-108), has been reported to reduce serum transaminase levels, increased levels of which are a symptom of chronic hepatitis C, and leads to viral seroconversion. Among the herbal components of Ninjinyoeito, Schisandra fruit and its lignan component, gomisin A, have shown protective effects against immunological hepatopathy [22]. We have previously reported that nine herbal extracts exhibit therapeutic anti-HSV-1 activity in a murine infection model [13]. Therefore, it would be logical to expect that the aforementioned crude drugs collected in Yunnan Province of China, exhibiting inhibitory potency, could be used to treat some cases of human viral infections.

The root bark of *T. hypoglaucum* (Y44) has been used in Chinese traditional medicine for hundreds of years to treat cancer and as an insecticide [23]. *Tripterygium hypoglaucum* is distributed mainly on rocky surfaces of lofty mountains in Tibet and Qing-hai provinces. In the last 30 years, many triterpenes, diterpenes, and sesquiterpenes have been isolated from *T. hypoglaucum* [24]. In this study, of the six compounds (compounds 1–6) isolated from *T. hypoglaucum*, demethylzeylasteral (compound 5) showed the highest inhibitory potency against HCV-RdRp with an IC_{50} value of 7.4 μM. In a comparison of HCV-RdRp inhibitory activity among compounds 5 and 6, and their derivatives, a carboxylic acid in the E-ring was deduced to be important for inhibitory



5 R_1=H, R_2=CHO, R_3=H, R_4=O
5a R_1=Ac, R_2=CHO, R_3=H, R_4=O
5b R_1=H, R_2=CH_2OH, R_3=H, R_4=O
5c R_1=Me, R_2=CHO, R_3=Me, R_4=O
6a R_1=H, R_2=Me, R_3=H, R_4=H_2

6

Fig. 1 Structures of compounds isolated from the root bark of *T. hypoglaucum* and their derivatives

activity. Moreover, aldehyde and hydroxyl groups in the A-ring were necessary structural elements for anti-HCV-RdRp activity. Alkaloids from *T. hypoglaucum* have recently been reported to induce apoptosis of HL-60 cells through c-myc and NF-κB signaling pathways [25]. Compound 5 has also been reported to inhibit both Ca^{2+} channel spermatogenic cells and a progesterone-induced sperm acrosome reaction in male mice [26]. Moreover, compound 5 has been reported to modulate tumor growth as well as neovascularization [27].

In conclusion, finding of compounds with HCV-RdRp-inhibitory activity from natural products will increase the importance of natural medicine research for anti-HCV therapeutic products. We report here, for the first time, HCV-RdRp-inhibitory activity of demethylzeylasteral (compound 5) from *T. hypoglaucum*. Due to HCV-RdRp-inhibitory activity of compound 5 and based on the previous research showing increased HCV-RdRp-inhibitory activity following alteration of functional groups in synthesized compounds [28], additional modification of the functional groups R1, R2, R3, and R4 (see Fig. 1) of compound 5 for leading to more potent HCV-RdRp-inhibitory substances would be a valuable step toward finding anti-HCV agents based on natural products.

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