Inhibitory effect of Tibetan medicinal plants on viral polymerases

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Abstract

For the purpose of development of novel anti-virus agents from ethnical drugs, we examined 76 traditional Tibetan medicines for inhibitory effect on two viral enzymes, reverse transcriptase (RTase) of HIV and RNA dependent RNA polymerase (RdRp) of HCV. Although 28 methanol extracts inhibited RTase more than 70 % at a concentration of 100 μg/ml, only 3 samples, T42 (藏青果, *Terminalia chebula* RETZ.), T46 (檳榔, *Areca catechu* L.) and T61 (紅檳榔), were found still inhibitory after eliminating the effect of tannins by addition of BSA in the enzyme reaction mixture. In the case of the RdRp, 7 extracts with IC_{50} values of less than 10 μg/ml contained less than 20 % tannins. The extract of *Rhodiola sacra*, whose IC_{50} for RTase was 25.9 μg/ml, was subject to phytochemical investigation. Out of 8 compounds isolated from the extract, daucosterol was found effective for RTase.

Key words HIV, HCV, reverse transcriptase, RNA-dependent RNA polymerase, enzyme inhibitor, Tibetan medicine.

Abbreviations HIV, human immunodeficiency virus; HCV, hepatitis C virus; RTase, reverse transcriptase; RdRp, RNA-dependent RNA polymerase; BSA, bovine serum albumin.

Introduction

HIV, human immunodeficiency virus, is a pathogen of AIDS, one of the most serious epidemics of today. Estimated death caused by AIDS during 2002 was 3.1 million worldwide.[1] The virus propagates itself by integration of its genome into that of a host. Genomic RNA of the virus is reverse-transcribed to the complementary DNA by a viral RNA dependent DNA polymerase, reverse transcriptase (RTase). The inhibitors of RTase are the most effective medicines against AIDS, at this moment. Nucleotide analogues (azidothymidine: AZT, didanosine: ddI, zalcitabine: ddC, stavudine: d4T, lamivudine: 3TC, abacavir: ABC) and non-nucleotide inhibitors (nevirapine : NVP, efavirenz : EFV, delavirdine: DLV) are approved by FDA for AIDS treatment. They are even more effective in combination with HIV protease inhibitors[2] (saquinavir: SQV, ritonavir: RTV, indinavir: IDV, nelfinavir: NFV). This combined treatment (highly active antiretroviral therapy, HAART)[3] has succeeded in elongation of the life span of AIDS patients. However, the appearance of mutated viruses which are resistant against these RTase inhibitors is inevitable, thus, new type of inhibitors must be constantly developed. Furthermore, about 9 % of individuals with HIV are coinfected with HCV, hepatitis C virus.[4] HCV has been identified as a causative virus of non-A non-B hepatitis. More than 170 million individuals worldwide are infected with HCV.[5] Besides the seriousness of a mass of carriers, type C hepatitis progresses into more serious diseases in high rate, such as cirrhosis and hepatocellular carcinoma.[5] HCV is also a RNA virus whose genomic RNA is served as mRNA for viral proteins and is also replicated by a virus specific RNA-dependent RNA polymerase (RdRp).[6] The newly synthesized RNA is subjected to encapsulation into viral capsule as genomic RNA of the virus of next generation. Although interferon therapy is the only way found effective for hepatitis C to some extent, more than 70 % of hepatitis C patients,

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especially these infected with a viral subgroup, Ib, which is most common in Japanese carriers, respond to the therapy very poorly.[7] Viral specific enzymes are thought to be the most appropriate target for antiviral agents. In many laboratories and pharmaceutical companies, seeking for inhibitors of well-characterized viral enzymes, RdRp and HCV protease, is currently progressing.[8] Mostly, a huge mass of randomly collected compounds and rationally synthesized substrate-related peptide derivatives are the candidates for the screening.

We have focused on naturally occurring products, especially, those which have been used as traditional medicines, for new resources of antiviral agents. In this paper, we report the result of inhibitory effect of traditional Tibetan medicines on two viral enzymes, RTase of HIV and RdRp of HCV. Since the Tibetan medicines are characterized by their original plants which grow in harsh growing conditions such as low oxygen pressure, intense ultraviolet rays and low temperature, they are expected to be a novel type natural resource with different characters from lowland plants. Our goal is discovery of crude drugs which have dual effect for both HIV and HCV from ethnical medicines.

Materials and Methods

Instruments : Ultraviolet spectrometer: Shimadzu UV-2200UV-VIS spectrophotometer. NMR : Varian Unity Plus 500 (^{1}H, 500 MHz, ^{13}C, 125 MHz), JEOL J NA - LAA 400 WB-FT (^{1}H, 400 MHz, ^{13}C, 100 MHz), Varian Gemini 300 (^{1}H, 300 MHz, ^{13}C, 75 MHz). Column chromatography : Fuji Silysia BW-820MH (silica gel), Pharmacia Sephadex LH-20, Organo Amberlite MB-3, Fuji Silysia Chromatorex-ODS DM-1020T (ODS). HPLC : Gilson HPLC system (pump: model 305 and 306, detector: 119UV/VIS detector). Preparative medium pressure liquid column chromatography (MPLC):Merck, Lichroprep Si 60 (size A). TLC: Merck precoated Silica gel 60F_{254} (0.25 mm), Merck RP-18 $F_{254}S$ (0.25 mm)

Crude drugs : The Tibetan crude drugs used in this report were collected in Qing-hai Province and Tibet Autonomous Region of China during 2000 for the JSPS Project of Overseas Survey on Ethnical Medicines. These specimens are deposited in the Herbarium of Materia Medica of Toyama Medical and Pharmaceutical University.

Preparation of methanol extracts : The crude drugs were dried and ground to a fine powder, and then extracted twice by refluxing in 100 ml methanol for 1.5 h. The extracts were passed through cotton filter and dried up under reduced pressure.

RTase assay : Recombinant HIV RTase was obtained from the Research Foundation of Microbial Disease of Osaka University. One unit is defined as the amount of enzyme that catalyzes incorporation of 1 nmol dTTP into acid-insoluble fraction within 10 min at 37 ℃. The enzyme reaction was conducted at 37℃ for 60 min in a reaction mixture which consisted of the following components; 50 mM Tris HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1.25 μg/ml poly(rA)-oligo(dT)$_{12\text{-}18}$, 250 nM dTTP, 100 nM [methyl-^{3}H]dTTP, and 0.05 unit/ml RTase. The extract dissolved in DMSO was added to the reaction mixture or DMSO to the control reaction at a concentration of 5 %. The reaction was carried out with or without BSA at a concentration of 0.5 mg/ml. The enzyme reaction was stopped by addition of 10 mM EDTA and the reaction mixture was applied onto a DE-81 filter paper (Whatman). The filter paper was rinsed 3 times with 5 % Na_2HPO_4 solution, once with water, once with ethanol and once ether, and then dried. The radioactivity of the filter paper was counted in a 3 ml of scintillation counter. Inhibition (%) was calculated as follows;

Inhibition (%)={1-(dpm sample/dpm control)} x 100

Adriamycin was used as a positive control.

RdRp assay : Recombinant HCV RdRp was prepared as reported.[9] The enzyme reaction was conducted at 25℃ for 60 min in a reaction mixture consisted of the following components; 20 mM Tris HCl (pH 8.3), 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 μg/ml poly (rA), 1 μg/ml oligo(U), 2 μCi ^{32}P-UTP, 10 μM UTP, and 90 μg/ml enzyme. The extract dissolved in 50 % DMSO was added to the reaction mixture or DMSO to the control reaction at a concentration of 2.5 %. The enzyme reaction was stopped by chilling on ice and the reaction mixture was applied onto a DE-81 filter paper. The filter paper was rinsed in the same way as RTase. The radioactivity of the filter paper was counted in a 3 ml of scintillation counter. Inhibition (%) was calculated as follows;

Inhibition (%)={1-(dpm sample/dpm control)} x 100

Adriamycin was used as a positive control.

Measurement of tannin content[10] : Methanol extract (0.2 mg/ml) 2 ml was mixed with 0.2 M phosphate buffer (pH7.0) 1 ml and 26 μg/ml methylene blue solution 2 ml. The mixture was left at room temperature for 30 min and centrifuged at 4000 rpm for 10 min. The absorbance at 660 nm of the supernatant was measured. The standard curve was obtained by using a solution of tannic acid (Nacalai Tesque Co.Ltd., Kyoto). Tannin content was calculated in terms of tannic acid.

Extraction and fractionation of Rhodiola sacra.: Underground part of *R. sacra* (3.3 kg) was ground and extracted with methanol (18 L, twice), and the solvent was removed under reduced pressure to obtain solid residue. The yield of the methanol extract was 447 g. The extract was dissolved in 90% methanol (1 L), and extracted with *n*-hexane (1 L, three times). Water phase was extracted with $CHCl_3$ (1 L, 3 times), and then applied onto Diaion HP 20 column (10×50 cm). The column was eluted stepwise with water, 50 %methanol and methanol. After removal of solvent, yields of the fractions were as follows: *n*-hexane fraction, 32.7 g, $CHCl_3$ fraction, 61 g, water fraction, 215 g, 50 % methanol elute 114 g, methanol elute 23.8 g. One hundreds gram of 50 % methanol fraction was fractionated by sillica gel column chromatography (sillica gel, 500 g) by stepwise elution of methanol-$CHCl_3$ solvent system (10:90 to 100:0) to 5 fractions (Fr.1 : 0.021 g, Fr.2 : 16.46 g, Fr.3 : 39.0 g, Fr.4 : 39.76 g, Fr.5: 2.0 g). Fr.2 (10 g) was further fractionated by ODS column chromatography eluted with MeOH: H_2O=1:1 to separate to 4 fractions (Fr. 2-1: 1.7 g, Fr.2-2: 5.82 g, Fr.2-3: 0.14 g, Fr.2-4: 0.8 g). Fr.2-1 was fractionated by ODS column chromatography with MeOH: H_2O=1:3, Sephadex LH-20 column chromatography and preparative TLC, and **4** and **6** were isolated. From Fr.2-2 (5.82 g), **7** and **8** were isolated by sillica gel column chromatography [MeOH: $CHCl_3$ (1:5)] and preparative HPLC [MeOH:H_2O (35:65)]. Fr.2-3 (0.14 g) was subjected to sillica gel column chromatography and MPLC (MeOH: $CHCl_3$=1:5), and **2** and **3** were isolated. Fr.2-4 (0.8 g) was fractionated on sillica gel column chromatography and MPLC, and **5** and **1** were obtained.

Characterization of isolated compounds:

Compound **1** : colorless amorphous powder. FAB-MS *m/z* : 577 $[M+H]^+$, ^{1}H-NMR (C_5D_5N, 500 MHz) δ 0.64 (3H, s, 18-H), 0.84 (3H, d, *J* = 7.0 Hz, 26-H), 0.86 (3H, d, *J* = 7.0 Hz, 27-H), 0.87 (3H, t, *J* = 7.0 Hz, 29-H), 0.91 (3H, s, 19-H), 0.96 (3H, d, *J* = 7.0 Hz, 26-H), 4.29 (1H, m, 3β-H), 3.90-4.60 (sugar protons), 5.05 (1H, d, *J* = 7.8 Hz, anomeric proton of glucose), 5.33 (1H, d, *J* = 5.1 Hz, 6-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of authentic daucosterol.

Compound **2** : colorless amorphous powder. ^{1}H-NMR (CD_3OD, 500 MHz) : δ 2.93 (2H, t, *J* = 7.0 Hz, 7-H), 3.75 (1H, dd, *J* = 10.0, 7.0 Hz, 8-H), 4.04 (1H, dd, *J* = 10.0, 7.0 Hz, 8-H), 4.29, 4.30 (1H each, both d, 1',1"-H), 7.13 -7.26 (5H, Ph). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of 2-phenylethyl α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside.[11]

Compound **3** : colorless amorphous powder. ^{1}H -NMR (CD_3OD, 300 MHz): aglycone moiety δ 2.93 (2H, t, *J* = 7.0 Hz, 7-H), 3.74 (1H, dd, *J* = 10.0, 7.0 Hz, 8-H), 4.09 (1H, dd, *J* = 10.0, 7.0 Hz, 7-H), 7.14 -7.25 (5H, Ph), sugar moiety δ 3.17 (1H, dd, *J* = 9.0, 7.0 Hz, 2'-H), 3.25-3.35 (3H, overlapped, 3',4',5'-H), 3.66 (1H, dd, *J* = 12.0, 5.0 Hz, 6'-H), 3.87 (1H, dd, *J* = 12.0, 2.0 Hz, 6'-H), 4.29 (1H, d, *J* = 7.0 Hz, 1'-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of 2-phenylethyl β-D-glucopyranoside.[12]

Compound **4** : colorless amorphous powder. ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.04 (2H, s, H-2, 6). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of authentic gallic acid.

Compound **5** : pale yellow powder. ^{1}H-NMR (CD_3 OD, 400 MHz): δ 0.87, 1.29 (3H each, both s, 8, 9-H), 1.19, 2.42 (1H each, both m, 6-H), 2.08 (1H, m, 5-H), 2.23 (1H, t, *J* = 5.6 Hz, 1-H), 2.29 (2H, m, 4-H), 3.72 (1H, dd, *J* = 11.6, 5.6 Hz, 6'-H), 4.00 (1H, dd, *J* = 12.5, 1.5 Hz, 10-H), 4.07 (1H, dd, *J* = 11.6, 2.2 Hz, 6'-H), 4.20 (1H, dd, *J* = 12.5, 1.3 Hz, 10-H), 4.26 (1H, d, *J* = 7.8 Hz, 1'-H), 4.30 (1H, d, *J* = 6.6 Hz, 1"-H), 5.58 (1H, br s, 3-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of sacranoside A.[11]

Compound **6** : colorless amorphous powder, ^{1}H -NMR (CD_3OD 400 MHz) : δ 1.80 (2H, m, 8'-H), 3.38-3.83 (Glc-H) , 2.61 (2H, t, *J* = 7.7 Hz, 7'-H), 3.55 (2H, t, *J* = 6.5 Hz, 9'-H) , 3.80 (3H, s, OMe) , 4.99 (1H, d, *J* = 7.0 Hz, Glc 1-H) , 5.50 (1H, d, *J* = 6.6 Hz, 7-H), 6.75

(1H, d, J = 8.1 Hz, 5-H), 6.81 (1H, br s, 2'-H), 6.83 (2H, dd, J = 8.1, 2.0 Hz, 6-H), 6.91 (1H, br s, 6'-H), 6.94 (1H, d, J = 2.0 Hz, 2-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectrum were identical with those of clemastanin A.[13]

Compound **7** : pale yellow powder. ^{1}H -NMR (CD_3 OD, 500 MHz): δ 6.75 (1H, d, J = 8.0 Hz, 5-H), 6.68 (1H, d, J = 2.0 Hz, 2-H), 6.65 (1H, dd, J = 8.0, 2.0 Hz, 6-H), 6.62 (1H, s, 5'-H), 6.17 (1H, s, 2'-H), 4.01 (1H, d, J = 11.5 Hz, 7-H), 3.80 (1H, dd, J = 13.5, 2.5 Hz, 9-H), 3.55 (1H, dd, J = 13.5, 4.0 Hz, 9-H), 3.79, 3.77 (3H each, both s, OCH_3), 3.78 (1H, d, J = 11.5 Hz, 9'-H), 3.57 (1H, d, J = 11.5 Hz, 9'-H), 3.21 (1H, d, J = 16.5 Hz, 7'-H), 2.60 (1H, d, J = 16.5 Hz, 7'-H), 2.02 (1H, ddd, J = 11.5, 4.0, 2.5 Hz, 8'-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of cycloolivil.[14]

Compound **8** : colorless amorphous powder, EI-MS m/z : 346 $[M]^+$, ^{1}H-NMR (CD_3OD, 500 MHz): δ 1.79 (2H, m, 8'-H), 2.55 (2H, t, 7'-H), 3.45 (1H, br q, J = 6.0 Hz, 8-H), 3.55 (2H, t, 9'-H), 3.74 (2H, dd, J = 10.5, 7.9 Hz, 9-H), 3.81 (3H, s, OMe), 5.48 (1H, d, J = 7.0 Hz, 7-H), 6.56 (1H, br s, 5-H), 6.60 (1H, br s, 2'-H), 6.75 (1H, d, J = 8.1 Hz, 6-H), 6.83 (1H, dd, J = 8.1, 2.0 Hz, 6'-H), 6.97 (1H, d, J = 2.0 Hz, 2-H). The other spectroscopic characteristics including ^{13}C-NMR (C_5D_5N, 75 MHz) spectra were identical with those of cedrusin.[13]

Results

Screening of RTase inhibitory extract from Tibetan crude drugs

Out of 76 Tibetan crude drug extracts (Table I) tested for inhibitory effect on HIV RTase, 28 extracts inhibited RTase more than 70 % at a concentration of 100 μg/ml. Among the constituents which were supposed to be contained in these crude drugs, tannins are ubiquitously contained in medicinal plants, and are known to

Table I List of crude drugs.

Extract NO.	Local name	family	Botanical name	Part used
T1	止瀉木子	Apocynaceae	*Holarrhena antidysenterica* Wall.ex.A.DC.	seed
T2	紫鉚子	Fabaseae	*Butea monosperma* (Lan.) Kuntze	mature seed
T3	芒果核	Anacardiaceae	*Mangifera indica* L.	seed
T4	蒲桃	Melastomatacceae	*Syzygium cumini* (L.) Skeels	fruit
T5	紫草茸		*Laccifer lacca* Kerr.	extraction
T6	黒種草子	Ranunculaceae	*Nigella glandulifera* Freyn (*N.sativa* L.)	mature seed
T7	香旱芹	Apiaceae	*Cuminum cyminum* L.	mature fruit
T8	草豆蔻	Zingiberaceae	*Alpinia katsumadai* Hayata	mature seed
T9	肉豆蔻	Myristicaceae	*Myristica fragrans* Houtt	aril
T10	蕃茇	Piperaceae	*Piper longum* L.	seed
T11	黒云香	Styracaceae	*Styrax tonkinensis* (Pierre) Craib ex Hart.	resin
T12	乳香	Burseraceae	*Boswellia carterii Birdw.* (*Shorea robusta* Gaertn.f.)	resin
T13	黄訶子肉	Combretaceae	*Terminalia chebula* Retz.	pericarp
T14	毛訶子肉	Combretaceae	*Terminalia bellirica* (Gaertn.) Roxb.	pericarp
T15	余甘子	Euphorbiaceae	*Phyllanthus emblica* L.	mature fruit
T16	檀香	Santalaceae	*Santalum album* L.	wood
T17	紫茉莉根	Nyctaginaceae	*Myrabilis himalaica* (Edgew.) Heim.	root
T18	沉香	Thymelaeceae	*Aquilaria sinensis* (Lour.) Gilg	wood
T19	邹木瓜	Rosaceae	*Chaenomeles speciosa* (Sweet) Nakai	fruit
T20	元胡	Papaveraceae	*Corydalis yanhusuo* W.T.Wang	tuber
T21	紫草	Boraginaceae	*Onosma hookeri* C.B.Clarke	root
T22	天竺黄	Poaceae	*Bambusa textilis* Mc.clure (B.arundinacea Willd)	resin
T23	冰片	Dipterocarpaceae	*Dryobalanops aromatica* Gaertn.f.	resin
T24	紅花	Asteraceae	*Carthamus tinctorius* L.	flower
T25	桂皮	Lauraceae	*Cinnamomum cassia* Presl	bark
T26	山沈香			wood
T27	藏菖蒲	Araceae	*Acorus calamus* L.	rhizome
T28	手掌参	Orchidaceae	*Gymnadenia conopsea* (L.) R.Br.	tuber

Extract NO.	Local name	family	Botanical name	Part used
T29	木鱉子	Cucurbitaceae	*Momordica cochinchinensis* (Lour.) Spreng.	seed
T30	馬銭子	Loganiaceae	*Strychnos nux-vomica* L. (*S. pierriana* A. W.Hill)	seed
T31	甘草	Fabaceae	*Glycyrrhiza uralensis* Fisch. (*G. glabra* L., *G. inflata* Bat.)	root
T32	寛筋藤	Menispermaceae	*Tinospora cordifolia* (Willd.) Hook.f.&Thomas. (*T. sinensis* (Lour.) Merr.)	tuber
T33	木香	Asteraceae	*Aucklandia lappa* Decne	root
T34	黒冰片			faces
T35	杜鵑花	Ericaceae	*Rhododendron anthopogonoides* Maxim.	flower,reaf
T36	大黄	Polygonaceae	*Rheum palmatum* L.	rhizome
T37	蒺藜	Zygophyllaceae	*Tribulus terrestris* L.	mature fruit
T38	香附子	Cyperaceae	*Cyperus rotundus* L.	rhizome
T39	骨砕補		*Drynaria sinica Diels* (*D.baronii* (Christ) Diels)	rhizome
T40	鬼臼果	Berberidaceae	*Sinopodophyllum emodii* (Wall.ex Royle) Ying	mature fruit
T41	天冬	Liliaceae	*Asparagus cochinchinensis* (Lour.) Merr.	tuber
T42	藏青果	Combretaceae	*Terminalia chebula* Retz.	fruit
T43	胡芦巴	Fabaceae	*Trigonella foenum-graecum* L.	mature seed
T44	党参	Companulaceae	*Codonopsis pilosula* (Franch.) Nannf.	root
T45	黄芪	Fabaceae	*Astragalus mongholicus* Bunge	root
T46	檳榔	Arecaceae	*Areca catechu* L.	mature seed
T47	黄精	Liliaceae	*Polygonatum cirrhifolium* (Wall.) Royle	rhizome
T48	白沙参	Campanulaceae	*Adenophora lilifolioides* Pax.et Hoffm	root
T49	角茴香	Papaveraceae	*Hypecoum leptocarpum* Hook.f.et Thomas. (*H. etectum* L.)	whole plant
T50	木通	Aristolochiaceae	*Aristolochia moupinensis* Franch. (*A.macrocarpa* C.Y.Wu et S.Y.Wu, *A griffithii* Thom. ex Duch.)	stem
T51	螃蟹		*Potamiscus loshingense* Wu	whole plant
T52	木棉花	Bombacacrae	*Bombax malabaricum* DC. (*B. ceiba* L.)	flower
T53	藏木香	Asteraceae	*Imura helenium* L. (*I. racemosa* Hook.f.)	root
T54	藏茜草	Rubiaceae	*Rubia wallichiana* Decne., *R. tibelica* Hook.f., *R. cordiholia* L.	aril part
T55	酸勝果	Myrisinaceae	*Embelia laeta* (L.) Mez.	fruit
T56	紫檀香	Fabaceae	*Pterocarpus indicus* Willd. (*P. santalinus* L.f.)	wood
T57	藏野姜	Zingiberaceae	*Hedychium spicatum* Ham.ex Smith	rhizome
T58	榼藤子	Fabaceae	*Entada phaseoloides* (L.) Merr.	mature seed
T59	腊腸果	Fabaceae	*Cassia fistula* L.	fruit
T60	藏商陸	Phytolaccaceae	*Phytolacca acinosa* Roxb.	root
T61	紅檳榔			seed
T62	棱子芹	Apiaceae	*Pleurospermum hookeri* (C.B.Clarke) *var. thomosonii* C.B.Clarke	root
T63	文冠木	Sapindaceae	*Xanthoceras sorbifolia* Bunge	stem
T64	風毛菊	Asteraceae	*Ixeris chinensis* (Thunb.) Nakai	whole plant
T65	川烏	Ranunculaceae	*Aconitum Carmichaeri* Debx.	tuber
T66	藏蔓菁			underground part
T67	天南星	Araceae	*Arisaema flavum (Forsk.) Shott.*	tuber
T68	白花竜胆	Gentianaceae	*Gentiana algida* Pall.	flower
T69	白芹艽花	Gentianaceae	*Gentiana straminea* Maxim.	flower
T70	甘粛雪霊芝	Caryophyllaceae	*Arenaria kansuensis* Maxim.	stem,rhizome
T71	懸鈎藤	Rosaceae	*Rubus biflorus* Buch.-Ham.ex Smith (*R. kokoricus* Hao.)	stem
T72	五綫緑繊花	Papaveraceae	*Meconopsis quintuplinervia* Regel	flower
T73	広酸棗	Anacardiaceae	*Choerospondias axillaris* (Roxb.) Burtt et Hill	mature fruit
T74	波棱爪子	Cucurbiaceae	*Herpetospermum pedunculosum* (Scx.) Bail.	seed
T75	美麗烏頭	Ranunculaceae	*Aconitum brunneum* Hand. -Mazz.	tuber
T76	紅景天	Crassulaceae	*Rhodiola sacra* S.H.Fu.	underground part

Table II Inhibitory activity of crude drug extracts on HIV RTase.

	IC_{50} (μg/ml)	Tannin content (%)
T1	11.1	10.4
T3	2.2	25.4
T4	10.4	12.4
T5	10.0	23.9
T8	27.7	19.8
T9	27.7	3.9
T13	0.1	23.9
T14	0.5	22.4
T15	1.1	19.3
T18	10.5	11.6
T19	7.3	3.9
T25	13.2	3.2
T34	9.4	3.7
T35	5.6	11.6
T36	0.4	19.1
T38	11.9	2.9
T39	2.9	6.7
T42	0.7	22.7
T46	0.1	24.7
T52	8.5	4.4
T55	26.9	19.8
T58	30.5	0.2
T59	1.4	17.2
T60	0.1	16.7
T61	0.8	16.8
T71	34.7	2.3
T73	20.0	6.4
T76	25.9	13.2

Value is mean. (n=3)

Table III Effect of albumin on HIV RTase -inhibitory activity of crude drugs.

	Inhibition of RTase (%) at 100 μg/ml	
	BSA (-)	BSA (+)
T1	94.5±4.27	20.2± 6.14
T3	96.6±1.10	17.4± 2.97
T4	99.8±0.26	-10.8±12.48
T5	96.3±2.89	24.7±19.72
T8	85.1±1.38	27.3±10.35
T9	94.4±1.58	30.2±12.86
T13	100.9±0.02	36.6± 4.78
T14	100.3±0.20	38.8±16.26
T15	100.5±0.40	12.0± 1.81
T18	98.4±0.52	34.4±10.51
T19	96.3±1.01	8.9±10.08
T25	94.4±2.08	10.7±44.65
T34	74.8±3.86	30.3±17.04
T35	93.6±1.26	-7.3±10.66
T36	98.5±0.44	35.4± 0.15
T38	88.9±1.51	41.0±11.38
T39	9.75±2.49	55.4±13.48
T42	**100.1±0.12**	**84.5±12.11**
T46	**100.1±0.16**	**93.5± 5.29**
T52	98.0±0.90	5.0±11.46
T55	94.7±2.28	36.8± 0.72
T58	80.0±4.36	5.3± 3.58
T59	101.5±0.12	32.0± 8.21
T60	100.7±0.31	15.2±12.05
T61	**101.4±0.08**	**70.0± 7.32**
T71	85.4±2.37	4.9± 8.61
T73	95.7±0.81	28.9± 4.54

Value is mean±S.D. (n=3)
Inhibitory effects of the above extracts measured in the presence (+) or absence (-) of 0.5 mg/ml BSA (bovine serum albumin).

Table IV Inhibitory activity of crude drug extracts on HCV RdRp.

	IC_{50} (μg/ml)	Tannin content (%)
T1	10.7	10.4
T3	9.8	25.4
T4	13.0	12.4
T5	10.8	23.9
T9	31.0	3.9
T13	1.8	23.9
T14	3.6	22.4
T15	5.7	19.3
T18	7.9	11.6
T19	9.8	3.9
T20	21.4	1.2
T21	35.6	23.1
T25	13.1	3.7
T27	39.4	0.0
T28	23.2	0.5
T34	30.2	3.7
T35	16.7	11.6
T36	9.3	19.1
T38	17.9	2.9
T39	9.1	6.7
T40	33.8	9.4
T42	0.9	22.7
T43	42.0	2.8
T46	2.6	24.7
T48	40.2	2.7
T52	26.9	4.4
T55	10.4	19.8
T57	24.9	2.2
T58	34.9	0.2
T59	9.7	17.2
T60	8.1	16.7
T61	11.3	16.8
T63	35.9	3.5
T71	33.4	2.3
T72	47.3	0.4
T73	28.7	6.4
T74	28.6	4.6

Value is mean. (n=3)

bound proteins non-specifically. The IC_{50} of 28 inhibitory extracts and tannin content, which was measured by the methylene blue analysis,[10] were determined as shown in Table II. Most of them, especially, whose IC_{50} was less than 1 μg/ml, had high tannin content, nearly 20 %. Whether their inhibitory effect was due to non-specific binding of tannin and proteins including the enzyme, the enzyme reaction was carried out in the presence of BSA. Three samples, T42, T46 and T61 were found still inhibitory (Table III).

Screening of RdRp - inhibitory extract from Tibetan crude drugs

The HCV RdRp is encoded in the *NS5B* region at the 5'-terminus of the virus genome. The recombinant RdRp was efficiently produced by bacterial system.[9] The inhibitory effect of the extracts on the RdRp was tested using the recombinant enzyme. Thirty-seven extracts inhibited RTase more than 80 % at a concentration of 100 μg/ml. Their IC_{50} and tannin content were determined as shown in Table IV. Twenty extracts had IC_{50} of less than 20 μg/ml. Three extracts, T42, T46 and T61 which were inhibitory for RTase inhibited RdRp efficiently.

Isolation of RTase inhibitory compounds from Rhodiola sacra

As shown in Table II, the extract of *Rhodiola sacra* (T76) effciently inhibited RTase. We investigated RTase inhibitory compound contained in the extract. The extract was fractionated and 8 compounds were isolated and were analyzed spectroscopically to determine the chemical structure. They were identified as **1**:

Table V HIV RTase -inhibitory activity of compounds isolated from *R.sacra.*

Compounds	Inhibition (%) at 100 μM
1	84.3± 5.5
2	58.9±26.9
3	25.8±25.1
4	18.2±10.5
5	28.3±20.5
6	8.0± 5.24
7	45.8±14.28
8	4.6± 8.69
(-)-Epigallocatechin gallate	91.0± 1.5
Kaempferol	50.2± 6.5
Caffeic acid	40.6± 8.0
Protocatechuic acid	39.7± 8.7
trans-p-Cumaric acid	38.2± 3.5
Umberiferone	36.6± 8.4
2-(4-Hydroxyphenyl)ethylalcohl	34.6± 3.6
Hydroquinone	30.6±35.1
4-Hydroxybenzoic acid	-3.9± 2.8
Suberric acid	-11.5±29.7
Adriamycin	92.2±8.90

daucosterol, **2**: 2-phenylethyl α-L-arabinopyranosyl-(1 → 6)-β-D-glucopyranoside, **3**: 2-phenylethyl β-D-glucopyranoside, **4**: gallic acid, **5**:sacranoside A, **6**: clemastanin A, **7**: cycloolivil, **8**: cedrusin. We examined the RTase inhibitory activity of these isolated compounds as well as the compounds which have been reported to be isolated from the crude drug, as shown Table V. Among the compounds, compound **1** and (-)-epigallocatechin gallate inhibited the enzyme efficiently.

Discussion

Most of the samples that had high tannins content inhibited RTase effectively, and the effect was diminished by BSA addition. Three exceptions, T42 (藏青果, *Terminalia chebula* Retz.), T46 (槟榔, *Areca catechu* L.) and T61(紅槟榔), were still inhibitory after eliminating the effect of tannins by addition of BSA in the enzyme reaction mixture. T42 is the unripe fruit of West Indian almond, and is mainly produced in Canton and Yunnan Province of China. In Tibet, it is used for eye diseases and diarrhea. T46 and T61 are the matured seeds of a plant of the palm family, and they are distributed in Tibet Autonomous Region, Canton and Yunnan Province. Their vermicide effect and antivirus activity have been reported.[15] These three crude drugs were also inhibitory on HCV RdRp. Their combined effect on these two viral polymerases could be the treatment for individuals who are coinfected with HIV and HCV.

T76 (聖地紅景天, *Rhodiola sacra*) is a typical Tibetan crude drug commonly used for hemostatics, treatment of a bruise and a burn, and is thought to stimulate blood circulation. It is getting popular as a health food and is sold in the market in Tibet. It is distributed mainly on the rocky surface of lofty mountains in Tibet and Qinghai Province. The isolation of the following compounds has been reported : flavonoids such as kaempherol, alkylated sugars, phenylpropanoids, *p*-hydroxybenzoic acid derivatives, β-sitosterol derivatives, monoterpenoids (sacranosides, kenposides), and condensed tannins and gallic acid.[11, 12, 16, 17] Among the eight compounds (**1** ~ **8**) which we isolated and identified, this is the first report of isolation of **6**, **7** and **8** from the crude drug. The constituents of the crude drug including those isolated by us showed moderate inhibitory effect for RTase. Compound **1**, the isolation of the compound has been reported by Qiu *et al.*,[16] showed the highest activity, 84 % inhibition at a concentration of 100 μM. Furthermore, most of the 76 Tibetan crude drugs examined here are used for preservation of hygiene and for long term medication. Their effect should be mild and have little side effect. The Tibetan medicines which were effective for two viral polymerases could be developed as supplement drugs over a long period of time.

和文抄録

民族薬物からの新たな抗ウイルス薬の開発を目指して，我々はチベット伝統薬物 76 種の HIV の逆転写酵素 (RTase) および HCV の RNA 依存 RNA ポリメラーゼ (RbRp) に対する阻害効果を検討した。これら76種のメタノールエキスのうち 28 検体が 100 μg/ml の濃度で RTase に 70% 以上の阻害効果を示したが，タンニンの効果を排除するため BSA 添加後も効果があったものはこのうち 3 検体のみであった。一方，同濃度で RdRp に 90% 以上阻害効果があり，またタンニンの含有率が10%以下のものは 8 検体であった。さらに RTase に対する IC_{50} が 5.9 μg/ml であった薬物の *Rhodiola sacra* のメタノールエキスの分画を行い 8 化合物を単離した。そのうち daucosterol に RTase 阻害作用を見い出した。

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