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Activity of compounds from Chinese herbal medicine *Rhodiola kirilowii* (Regel) Maxim against HCV NS3 serine protease

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Abstract

Treatment of the chronic hepatitis C virus (HCV) infection is an unmet medical need, and the HCV NS3 serine protease (NS3-SP) has been used as an attractive target of antiviral screening against HCV. To find naturally chemical entities as lead compounds from which novel anti-HCV agents could be developed, bioassay-guided fractionation and isolation were performed on a crude ethanol extract from rhizomes of the Chinese medicinal herb *Rhodiola kirilowii* (Regel) Maxim using column chromatography (CC) techniques and in vitro inhibitory activity against HCV NS3-SP. The partition of the extract between water and different organic solvents led to the isolation and identification of 12 compounds in the ethyl acetate part which proved to be the most active. These compounds were tested for in vitro activity against HCV NS3-SP, among which four (−)-Epicatechin derivatives: 3,3′-Digalloylproprodelphinidin B2 (Rhodisin, **1**); 3,3′-Digalloylprocyanidin B2 (**2**); (−)-Epigallocatechin-3-O-gallate (EGCG, **3**); and (−)-Epicatechin-3-O-gallate (**4**, ECG) represented the most potent ones with IC_{50} of 0.77, 0.91, 8.51, and 18.55 μM, respectively. Salidroside, the commonly known compounds, together with the other compounds showed no activity up to 100.0 μM. Methylation and acylation of the hydroxyl groups of **1–4** caused a decrease of activity. Cell viability and secreted alkaline phosphatase (SEAP) activity assays with **1–4** revealed little if any toxicity. These nonpeptide inhibitors of HCV NS3-SP might serve as potential candidate anti-HCV agents.

Keywords: Antiviral; HCV NS3-SP; (−)-Epicatechin derivative; *Rhodiola kirilowii* (Regel) Maxim

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease which infects more than 170 million persons worldwide, often leading to cirrhosis, hepatic failure, and hepatocellular carcinoma. The current approved therapy is confined to interferon-α (INF-α) and ribavirin, but the overall chances of cure is below 50%. Still there is no preventive vaccine available. Hence, there remains the urgent need to find therapeutic agents. HCV is an enveloped virus with positive-strand RNA genome encoding a polyprotein. The polyprotein is cleaved by NS3 serine protease (NS3-SP) at four junctions (NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B) in the nonstructural part of this protein, NS4A being a cofactor of the protease (Blight et al., 1998). The NS3/4A serine protease has been well-characterized biochemically, and the crystal structure has been solved by several groups, making it an attractive target for antiviral drug discovery and drug design (De Francesco and Steinkuhler, 2000; De Francesco et al., 2003).

Rhodiola consists of a number of species that are popular in traditional Chinese Tibetan medicinal herbs (Hongjingtian in Chinese). It has been reported to possess properties of resisting anoxia, microwave radiation and fatigue. As a drug of "source of adaptation to environment" in traditional chinese medicine (TCM), it has been used in divers, astronauts, pilots and mountaineers to enhance the body's ability to survive in adverse environments. The major effective constituent of this herb is known as Salidroside (Li and Chen, 2001).

As a part of our continuing searching for natural inhibitors of NS3-SP, we screened over 40 ethanol extracts of Chinese medicinal herbs (data no shown). One of these extracts from the rhizomes of *Rhodiola kirilowii* (Regel) Maxim was active in NS3-SP assay. Therefore, we traced the efficient constitution of the extract through bioassay-guided fractionation and isolation

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procedures (Tan et al., 1991). Here, the identification of the compounds in the extract and their activities against HCV NS3-SP is reported.

2. Materials and methods

2.1. Plant material

The rhizomes of *R. kirilowii* were purchased from the Qinghai Institute of Tibetan Medicines (Qinghai Province, China) where a voucher specimen was deposited.

2.2. Cells

Cos-7(NS3/4A-SEAP) cell lines used for cytotoxicity and secreted alkaline phosphatase (SEAP) activity assays were obtained from the Key Laboratory of Medical Molecular Virology (Shanghai Medical College, Fudan University, Shanghai, China). The cells were transfected with pCI-neo-NS3/4A-SEAP chimeric plasmid and were cultured in Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/ml) and streptomycin (100 μg/ml), supplemented with 10% fetal calf serum. Their SEAP activity was determined by a colorimetric assay according to the authors' recommendation (Mao et al., 2003).

2.3. Expression and purification of HCV NS3-SP domain

The recombination plasmid pMANS34NSH containing MBP-NS3-SP was constructed and transformed into *E. coli* JM109. HCV NS3-SP domain was expressed and purified following the protocol described in the literature (Kakiuchi et al., 1995).

2.4. NS3-SP activity assay

The activity of HCV NS3-SP in the expressed domain was assayed by enzyme-linked immunosorbent assay (ELISA) according to the reported method (Takeshita et al., 1997). A peptide substrate with an acetylgroup at the N-terminus and a biotin at the C-terminus was hydrolyzed by the protease into a product with a free amino moiety at N-terminus. The product was immobilized and the free amino moiety was analyzed.

For the inhibitory activity assays, aliquots of each sample 10 mg/ml (DMSO) were diluted to 1.0, 0.1, 0.01, 0.001 mg/ml (DMSO), respectively. Peptide substrate, NS3-SP and inhibitor were added and incubated at 37 °C for 15 min. The relative inhibitory rate (%) was calculated as $[(c-a)-(b-a)]/(c-a)\times 100$, where a: DMSO + substrate; b: enzyme + inhibitor + substrate; c: DMSO + enzyme + substrate. The inhibitory activities were shown as average values in duplicates obtained from two independent experiments. Each value of IC_{50} was the concentration of a tested sample required to inhibit up to 50% of protease activity as compared with the substrate control. Naphthoquinone (Sigma, St. Louis, MO) was used as a positive control drug in the anti-HCV NS3-SP assays (Zuo et al., 2005).

2.5. Antiviral-guided extraction and fractionation of the rhizome extract from R. kirilowii

We conducted the assay of the HCV NS3-SP activity as an antiviral guideline (Takeshita et al., 1997). The powdered rhizomes of *R. kirilowii* (1 kg) were extracted with ethanol (80%, 4000 ml × 3) at room temperature and concentrated to dryness *in vacuo* to afford a crude extract (190 g). The crude extract was suspended in deionized water and successively partitioned with petroleum ether (60–90 °C), chloroform, ethyl acetate and *n*-butanol (250 ml × 3 each). The organic layers were concentrated to dryness *in vacuo* to afford four parts (P, C, E and B) which were assayed for inhibition of HCV NS3-SP activities, respectively (data no shown).

2.6. Isolation and identification of compounds from the active part

Part E (10 g), the most active antiviral fraction (IC_{50} 50 μg/ml) from the fractionation with ethyl acetate, was passed through a polyamide resin (200 mesh, Zhejiang, China) column (Collins et al., 1998) and eluted with aqueous acetone (80%). The eluate was evaporated of solvent to afford a

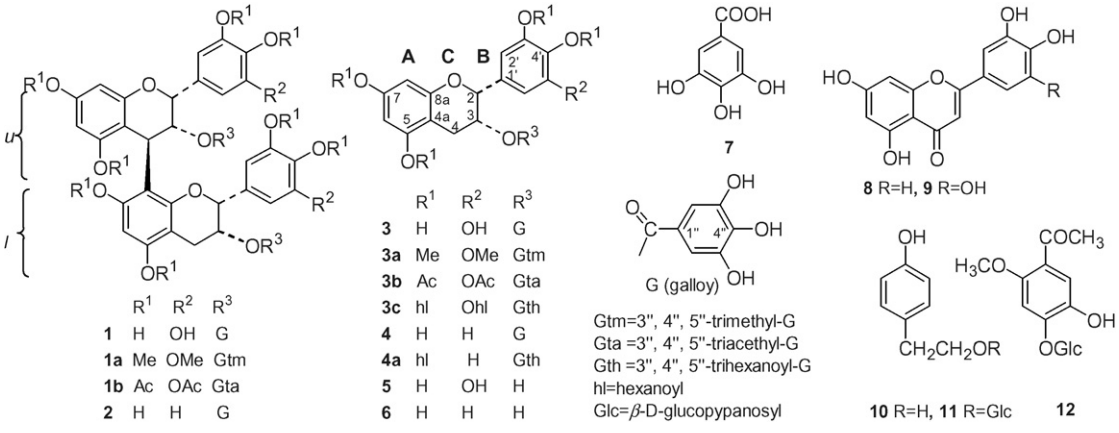

Fig. 1. Chemical structures of compounds **1–12**.

brown powder Ea (7.5 g), with no decrease of activity (data no shown). The powder was subjected to column chromatography (CC) *in vacuo* (VLC) with silica gel (300–400 mesh, Qingdao, China), eluted by gradient systems of petroleum ether–chloroform–methanol–formic acid (10:2:0:0–0:8:3:0.05) to afford five sub-parts (Ea-1–5). Repeated VLC of the sub-parts Ea-2–4 with silica gel (500 mesh) to afford compounds **7**, **8** and **10** (from Ea-2); **5**, **6**, **11** and **12** (from Ea-3); **3**, **4** and **9** (from Ea-4). The sub-part Ea-5 (1.2 g) was repeated VLC on polyamide resin (200 mesh, Zhejiang, China) with solvent systems of ethyl acetate–methanol–water (8:2:1–5:2:1) and ethanol–acetone–water (6:3:1) to afford two sub-parts Ea-51 and Ea-52. The latter (400 mg) was further subjected to CC on Sephadex LH-20 (Phamacia), eluted with ethanol–acetone–water (4:4:1 and 6:0:5) to afford compound **1** (40 mg) and **2** (65 mg), respectively (Fig. 1).

Compounds **1–4** were obtained as pale brown amorphous powders. The polyphenolic property of **1–4** was demonstrated by a dark blue color reaction with $FeCl_3$ reagent (5% in ethanol). Their structures were identified mainly by spectral analysis. Briefly, the negative ESI-TOF-MS showed molecular ion peaks at *m/z* 913, 881, 457 and 441 ($[M-H]^-$), corresponding to the molecular formulae $C_{44}H_{34}O_{22}$, $C_{44}H_{34}O_{20}$, $C_{22}H_{18}O_{11}$ and $C_{22}H_{18}O_{10}$, respectively. Compounds **1** and **2** were two dimers of compounds **3** and **4**, respectively. The typical NMR spectral data (Table 1) showed H and C resonance signals of characteristic 2, 3-*cis* configuration of flavan-3-ol in the upper and lower units, i.e., δ_H5.97–6.01 (δ_C95.9–107.8) ppm which were attributed to H(C)-6, 8 (A-ring), δ_H2.85–5.54 (δ_C 26.6–78.7) ppm to H(C)-2–4 (C-ring), and δ_H6.49–6.83 (δ_C 106.3–146.7) ppm to H(C)-2′, 5′-6′ (B-ring). Their galloyl signals were revealed by δ_H6.94–6.98 and δ_C 110.2–146.9 (H(C)-1″-3″), and δ_C167.4–167.7 (C=O) ppm. The C-4β →C-8 linkages of the two flavan-3-ols in compounds **1** and **2** were also suggested by the significant changes of all corresponding chemical shifts of H(C)-4 and -8. The rest ^1H and ^{13}C NMR chemical shifts and other spectral data of compounds **1–4** were consistent with those in the literature (Nonaka et al., 1981; Porter et al., 1982; Sun et al., 1988), and were further demonstrated by 2D NMR correlations (HSQC and HMBC). Therefore, these compounds were identified as: 3,3′-Digalloylproprodelphinidin B2 (Rhodisin, **1**); 3,3′-Digalloylprocyanidin B2 (**2**); (−)-Epigallocatechin-3-O-gallate (EGCG, **3**); and (−)-Epicatechin-3-O-gallate (**4**, ECG), as shown in Fig. 1.

Other compounds isolated from the active ethyl acetate part were (−)-Epigallocatechin (**5**) (−)-Epicatechin (**6**), Gallic acid (**7**) (Cai et al., 1991; Sun et al., 1988), Luteolin (**8**, Youssef and Frahm, 1995), Tricetin (**9**, Campos et al., 1997), Tyrosol (**10**) and Salidroside (**11**, Ayer et al., 1986), and Rodiolinozide (**12**, Jakupovic et al., 1991). The chemical structures were iden-

Table 1
Typical ^1H and ^{13}C NMR chemical shifts of compounds **1–4** (δ: ppm in CD_3OD)

No.		**1**		**2**		**3**	**4**
	–	Upper	Lower	Upper	Lower	–	–
2	H	5.54[a]	5.43[a]	5.52[a]	4.87[a]	5.52[a]	5.51[a]
	C	75.9	78.3	76.4	78.7	78.6	78.6
3	H	5.12[a]	5.44[a]	5.07[a]	5.42[a]	5.42[a]	5.41[a]
	C	74.4	68.9	74.1	69.0	69.9	70.0
4	H	4.86[a]	2.87[a]	4.86[a]	2.88[a]	2.86[a]	2.85[a]
	C	33.4	26.6	34.6	29.5	26.8	26.9
6	H	5.97[b]	5.96[b]	5.97[b]	5.94[b]	5.95[b]	5.95[b]
	C	96.6	96.1	96.0	96.3	95.9	95.9
8	H	6.01[b]	–	6.01[b]	–	5.95[b]	5.95[b]
	C	96.9	107.3	96.3	107.8	96.6	96.6
2′	H	6.52[b]	6.52[b]	6.51[b]	6.51[b]	6.50[b]	6.49[b]
	C	106.3	106.7	115.2	115.9	106.9	115.1
5′	H	–	–	6.68[a]	6.68[a]	–	6.68[a]
	C	145.1	143.5	115.9	115.2	146.7	116.0
6′	H	6.52[b]	6.54[b]	6.83[a]	6.83[a]	6.50[b]	6.81[a]
	C	106.3	106.7	119.8	119.8	106.9	119.4
1″	H	–	–	–	–	–	–
	C	121.7	121.4	121.7	121.5	121.6	121.5
2″	H	6.98[b]	6.98[b]	6.96[b]	6.96[b]	6.94[b]	6.94[b]
	C	110.5	110.6	110.3	110.4	110.3	110.2
3″	H	–	–	–	–	–	–
	C	146.9	146.3	146.2	146.2	146.3	146.3
C=O		167.4	167.5	167.7	167.7	167.7	167.6

[a] Referred to multiple proton resonance signals.
[b] Referred to single or broad single proton resonance signals.

Table 2
Inhibitory activities of the purified compounds and their derivatives against NS3-SP

Compounds	Inhibition (%)	IC_{50} [c] (μM)
1	63.2[a], 81.7[b]	0.77 ± 0.06*
2	60.8[a], 80.0[b]	0.91 ± 0.14*
3	27.8[a], 85.8[b]	8.51 ± 2.40*
4	55.7[a], 90.8[b]	18.55 ± 1.22*
1b	32.4[a], 80.6[b]	25.96 ± 1.56*
3b	35.3[a], 85.4[b]	44.55 ± 2.18*
3a	34.0[a], 82.3[b]	56.47 ± 4.65*
4a	33.1[a], 53.1[b]	70.06 ± 4.11*
[d]**PC**	73.5[a], 87.2[b]	8.40 ± 1.12*

[a] Inhibition (%) at 10 μg/ml
[b] Inhibition (%) at 100 μg/ml
[c] Tested at a maximum concentration of 100 μM, and IC_{50} of compounds **1a**, **3c**, **5–8** and **10–12** were >100 μM
[d] **PC** referred to positive control ([1,4] Naphthoquinone)
* Expressed as the mean ± S.D. from three replicate experiments. Student's *t*-test for unpaired data was used for statistical comparison, $P < 0.05$.

tified by spectrometry (data not shown) and *co*-TLC with the authentic samples (NICPBP, China). Six chemical derivatives of compounds **1–4** were prepared by routine procedures of methylation (**1a**, **3a**), acetylation (**1b**, **3b**) and hexanoylation (**3c**, **4a**), respectively (Kashiwada et al., 1986a, 1986b; Nonaka et al., 1981; Zuo, 2003; Fig. 1).

All the above mentioned compounds were subjected to assay of their inhibitory activities against HCV NS3-SP (Table 2).

2.7. Cytotoxicity assay

Cos-7(NS3/4A-SEAP) cells were cultured in DMEM medium and transfected with recombinant plasmid DNA following the protocol reported in the literature (Mao et al., 2003). Cell suspensions (0.2 ml of 2×10^4 cells/ml) were prepared in a 96-multi-well plate and incubated at 37 °C in an atmosphere of 5% CO_2 overnight. Drug-containing medium (0.2 ml, respectively) at five concentrations (each with five times dilution sequentially) was added to the cell culture, and the blank (DMSO) and positive control were treated with medium containing equivalent concentrations, respectively. After 24 h of incubation, 0.01 ml of MTT was added to each well and the culture was incubated for a further 4 h and the number of surviving cells was counted. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density (OD) of each well at 570 nm, using an MRX multiwell spectrophotometer. The cytoxicity index (CI%) was calculated according to the equation $CI\% = [1-(T/C)] \times 100\%$, where T and C represent the mean optical density of the treated group and vehicle control group, respectively (Table 3). Accordingly, the CI% of the dose response curve was drawn with Origin Pro 7.0 (OriginLab) (Fig. 2). All experiments were repeated at least three times. The median cytotoxic concentration (CC_{50}) was displayed as the concentration of the sample that reduced the number of viable cells to 50% of the cell control through the OD values of viable cells in comparison with non-viable cells. Each CC_{50} value was calculated by Logit model.

Table 3
Cytotoxicity of compounds **1–4**

No	CC_i[a]	CC_{50}[b]	SI[c]
1	295.17	513.81	667
2	328.53	419.15	461
3	1319.89	1788.95	210
4	1718.02	–[d]	–

The cytotoxicity was measured with the MTT assay.
[a] The initial cytotoxic concentration of target cell (Cos-7) in M.
[b] The 50% cytotoxic concentration of target cell (Cos-7) in M, calculated with Logit model.
[c] Selectivity index (SI) = CC_{50}/IC_{50}.
[d] Not determinable.

2.8. SEAP activity assay

Cos-7 (NS3/4A-SEAP) cells were seeded into a 6-well NUNC multidish at a density of 10^5 cells/ml in 3 ml medium and cultured overnight at 37 °C in a 5% CO_2 incubator. The overnight culture media were changed with fresh one containing the drug each at five diluted concentrations from 1 to 5 mg/ml. The cells without the compounds treatment were set as control (concentration-1). After 24 h, the media were collected for the colorimetric assay for SEAP activity according to the Supplier's recommendation (InvivoGen™, San Diego; Berger et al., 1988; Mao et al., 2003). Briefly, 20 ml heat-treated (at 65 °C for 5 min) medium was adjusted to 1 × SEAP assay buffer (1.0 M diethanolamine pH9.8, 0.5 mM $MgCl_2$, 10 mM L-homoarginine) in a final volume of 200 ml and prewarmed to 37 for 10 min in a 96-well flat-bottom culture dish. Twenty mililiters of prewarmed 120 mM *p*-nitrophenylphosphate dissolved in SEAP assay buffer were then added with mixing. The A_{405} of the reaction mixture was read in a BIO-RAD (Benchmark) microplate reader at 5-min intervals. The change in absorbance was plotted and the maximum linear reaction rate was determined. The SEAP activity was expressed in milliunits (mU) per ml. One mU equals an increase of 0.04 A_{405} units per min. Each SEAP assay was performed in triplicate (Fig. 2).

3. Results

3.1. Inhibition of compounds *1–12* against NS3-SP activity

Table 2 displayed inhibitory activities of the twelve isolated compounds and chemically prepared derivatives against NS3-SP. The prominent four active compounds were compounds **1–4**, with an increasing order of IC_{50} values as 0.77, 0.91, 8.51 and 18.55 μM, respectively. Over half of the tested compounds (**1a**, **3c**, **5–8** and **10–12**) did afford inhibition at concentrations up to 100 μM (data not shown). Compounds **1** and **2** represented the two most active entities. Both the methylation and acylation of the free hydroxyl groups resulted in a remarkable decrease of activity.

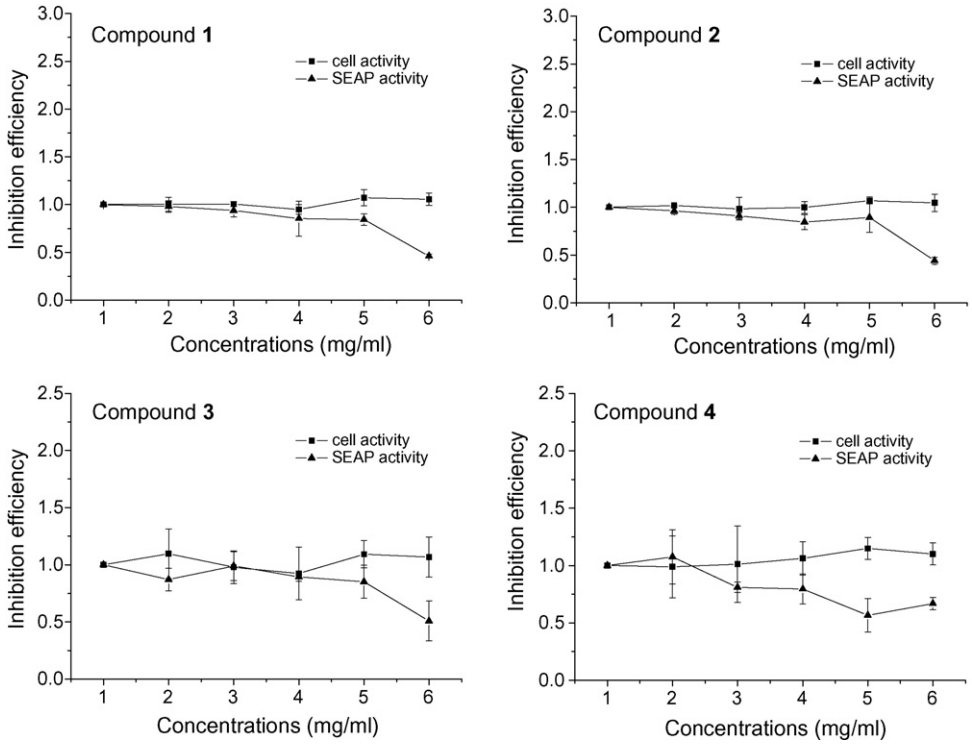

Fig. 2. Cytotoxity and SEAP activity of Cos-7 (NS3/4A-SEAP) cells treated with compounds **1–4**. The viability of cells was measured with the MTT assay. The SEAP activity was measured with a colorimetric assay according to manufacturer's recommendations (InvivoGen™, San Diego, USA). The concentrations of Nos.1–6 in the *x*-axis were of 0, 1, 2, 3, 4, 5 mg/ml, with No.-1 as negative control and No. -6 at the maximum concentration, respectively. The value of 1.0 in the *y*-axis represented the negative control (no treatment), the other values were calculated in comparison with 1.0. Each assay was performed in triplicate. The plots were made with OriginPro 7.0.

3.2. Cytotoxicity of compounds *1–4*

To explore the potential use of the active compounds **1–4** as antiviral agents, we tested their possible in vitro cytotoxicity by the MTT assay. As shown in Table 3 and Fig. 2, neither of them showed any cytotoxic effects on Cos-7 cell cultures at concentrations lower than 295 μM. The 50%-cytotoxic concentrations (CC_{50}), i.e., the concentration corresponding to a 50% cytotoxic effect after 24 h of cell treatment varied from 667 to 210 μM from compound **1** to compound **4**. Similar low cell viability interference of EGCG (**3**) has been found in different mammalian cell lines (Yamaguchi et al., 2002).

3.3. SEAP activity of compounds *1–4*

As the protease activity of NS3 was considered to be reflected by the activity of secreted alkaline phosphatase (SEAP) in the culture media of pCI-neo-NS3/4A-SEAP chimeric plasmid expressed Cos-7 cell lines (Mao et al., 2003), we investigated the effects of compounds **1–4** on SEAP activity with the cell lines by a colorimetric assay (InvivoGen™, San Diego). The results showed that they had little inhibitory activity against SEAP, despite the potent effect on NS3-SP (Fig. 2).

4. Discussion

The present studies analyzed possible NS3-SP targeted anti-HCV activity of compounds from the rhizome extract of *R. kirilowii*, a special medicinal plant indigenous to the high altitude of Qinghai-Tibet Plateau in China. Among the twelve isolated and identified compounds obtained from the active fraction, four polyphenols derived from 2, 3-*cis* flavan-3-ol or (−)-Epicatechin represented the most active entities (IC_{50}: 0.8–19 μM). The other compounds including conventional constituents of Salidroside and Tyrosol (Li and Chen, 2001) from the plant species showed activity from over one hundred to thousands of micromoles (data not shown). For all the tested (–)-Epicatechins, the inhibition of the protease decreased in the order galloylated (−)-Epi (gallo) catechin dimers > galloylated (−)-Epi (gallo) catechin monomers ≫ (−)-Epi (gallo) catechin monomers. Gallic acid (**7**) which is an esteration unit in these compounds only had an IC_{50} value as high as 1710 μM (Zuo et al., 2005) and (−)-Epicatechin (**6**) had an IC_{50} value of 261 μM (data no shown). In another assay, we also found that (−)-Epiafzelechin whose hydroxyl group is only one in B-ring, exerted an IC_{50} > 3650 μM (33.3% inhibition at 1000 μg/ml). These findings revealed two important points of structure-activity relationships of the anti-HCV natural entities: polyphenolic property of the compounds showed potent activity and galloylation added to the potency. The results also showed that the 5′-OH in B-ring plays a minor role in the anti-HCV activity, which has also been observed for the antiviral study of catechins on influenza virus (Song et al., 2005).

The polyphenols we present herein could be chemically classified as constituents of condensed tannin, which are different from the other promising active type of polyphenolic com-

pounds, *i.e.*, hydrolysable tannin in a previous paper (Zuo et al., 2005). Compound **9**, a flavonoid contains a small amount of phenolic hydroxyl groups, showed a little activity (IC_{50}: 121.1 μM, data no shown).

The HCV NS3-SP belongs structurally to the trypsin superfamily. A peculiar feature of the enzyme is the requirement of zinc ion for activity. Zn^{2+} was initially proposed to have an essential structural role, as its removal caused unfolding and precipitation of the protein (De Francesco et al., 1996; De Francesco et al., 1998). Haslam suggested that polyphenols probably exert certain of their roles by virtue of three distinctive *general* characteristics of their complexation with metal ions, antioxidant and their ability to complex with other molecules including macromolecules such as proteins and polysaccharides (Haslam, 1996). Hence, the potent anti-NS3-SP activities of the compounds might be partly attributed to their complexation with zinc ion in the protease. This kind of ability would be reinforced by the increasing of free hydroxyl groups in the structure, and *vice versa*, weakened by the removal or blockade of the free hydroxyl groups. Once the hydroxyl groups were methylated or acylated (compounds **2a–4c**), their activities decreased markedly. So the free hydroxyl groups in these compounds played an important role in the inhibitory effect on NS3-SP (Haslam et al., 1989).

With respect to molecular size of the active entities, moderate molecular weights of 500–1000, like for galloylated (−)-Epi (gallo) catechin monomers or dimmers, as demonstrated in our current studies, seemed to be appropriate for complexation with the biological macromolecules (the protease), because the detannining procedure, with which trimers, tetramers or polymers were removed from the ethyl acetate extract by passing through polyamide column, did not reduce the activity (data not shown). This is similar to those previous results of the galloylated glucose (Zuo et al., 2005). However, the activity of trimers and tetramers of galloylated catechins remains to be clarified.

The effect that linkage of a galloyl group at C-3 of (−)-Epigallocatechin (**5**) and (−)-Epicatechin (**6**), together with their dimers gave increased antiviral activities was also observed in the evaluation of anti-HIV and anti-HSV effect (Hashimoto et al., 1996; Vlietinck et al., 1998; De Bruyne et al., 1999a; Yamaguchi et al., 2002).

It should also be noted that, potent as direct inhibition against NS3-SP in the recombination plasmid pMANS34NSH, the (−)-Epicatechins showed not only little cytotoxicity but also very weak inhibition of SEAP activity in the Cos-7 cell lines which were transfected with chimeric plasmid of pCI-neo-NS3/4A-SEAP and the SEAP was fused in-frame to the downstream of NS4A/4B cleavage site. The protease activity of NS3 was taken as to be reflected by the activity of SEAP in the cells' culture media. The rationale for this system was based on the assumption that the secretion of SEAP protein into the culture media depends on the cleavage between NS4A protein and SEAP protein by HCV NS3-SP (Mao et al., 2003). However, after treatment with the compounds, the decrease of NS3-SP activity was not likely to be in line with the decrease of SEAP activity in this study. Therefore, the results of SEAP activity assay for screening anti-NS3-SP entities deserves for further investigation.

Compared with the synthetic HCV NS3-SP inhibitors, non-peptidic natural compounds are few (Chu et al., 1999; Hussein et al., 2000; Hegde et al., 2003; Duan et al., 2004; Zuo et al., 2005). Discovery of natural antivirals against the virally encoded enzymes remains to be a great effort (Huang et al., 2006). Up to now, although the antiviral and antimicrobial effects of catechin derivatives have been widely recognized (De Bruyne et al., 1999b; Yamaguchi et al., 2002; Crespy and Williamson, 2004), their inhibition against HCV NS3-SP was reported for the first time. The novel antiviral evidences of compounds from *R. kirilowii* provided interesting naturally chemical clue for the development of potential anti-HCV agents.

In conclusion, the low cytotoxic galloylates of (−)-Epicatechin and (−)-Epigallocatechin and their dimers has been found to exhibit in vitro antiviral activity against HCV NS3-SP with the dimers to be the most active.

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