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Anti-HIV-1 activities of extracts from the medicinal plant *Rhus chinensis*

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Abstract

Rhus chinensis, a species used in folk medicine by Chinese native people, the anti-HIV-1 activities of the petroleum ether, ethyl acetate, butanol and aqueous extract of *Rhus chinensis*, named as RC-1, RC-2, RC-3 and RC-4, respectively, was evaluated. The petroleum ether extract RC-1 can inhibit the syncytium formation and HIV-1 p24 antigen at non-cytotoxic concentrations, the 50% effective concentration (EC_{50}) were 0.71 and 0.93 μg/ml, respectively. The therapeutic index (TI) was about 100. RC-1 had no activity on inhibiting HIV-1 recombinant RT and HIV-1 entry into host cells. Results showed that RC-1 was effective against HIV-1 and *Rhus chinensis* would be a useful medicinal plant for the chemotherapy of HIV-1 infection. RC-1 might inhibit the post steps or target the new sites of HIV-1 replication.

Keywords: *Rhus chinensis*; HIV-1; Anti-HIV activity; Botanicals

1. Introducion

Combination therapy of anti-HIV drugs now available has improved the life quality and span of HIV/AIDS patients. Emergence of HIV drug resistance, side effects and the need for long-term antiretroviral treatment are the main causes for the failure of ART. Continuous development of new anti-HIV agents, targets and therapy appeared to be inevitable.

Natural products as the most consistently successful source in drug discovery, may offer more opportunities to find anti-HIV drugs or lead compounds. Many compounds with anti-HIV-1 effect have been screened out from natural products and discovered to inhibit HIV at nearly all stages of viral life cycle (Wang et al., 2004a). They include alkaloids, sulphated polysaccharides, polyphenolics, flavonoids, coumarines, phenolics, tannins, triterpenes, lectins, phloroglucinols, lactones, iridoids, depsidones, *O*-caffeoyl derivatives, lignans, ribosome inactivating proteins, saponins, xanthones, naphthodianthrones, photosensitisers, phosholipids, quinines and peptides (Ng et al., 1997; Vlietinck et al., 1998; Yang et al., 2001). Natural products provide a large reservoir for screening of anti-HIV agents with novel structure and anti-viral mechanism because of their structural diversity. A variety of natural products have been found to inhibit unique enzymes and proteins crucial to the life cycle of HIV including efficient intervention with the reverse transcription process, virus entry, the integrase and protease (De Clercq, 2000; Cos et al., 2004). But mechanism of anti-HIV activities of many more natural products is unknown.

In some countries, traditional medicine is used to meet the primary health care need and to treat AIDS patients (Harnett et al., 2005; Zhang et al., 2005). In China, the government has strengthened research and development of ART through traditional Chinese medicine. It has funded an intervention program

Abbreviations: AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; AZT, zidovudine; CC_{50}, 50% cytotoxic concentration; CPE, the cytopathic effect; DS, dextran sulfate; EC_{50}, 50% effective concentration; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RC-1, petroleum ether extract of *Rhus chinensis*; RC-2, ethyl acetate extract of *Rhus chinensis*; RC-3, butanol extract of *Rhus chinensis*; RC-4, water extract of *Rhus chinensis*; $TCID_{50}$, the 50% HIV-1 tissue culture infectious dose

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that supports hospitals to trial Chinese traditional and herbal drugs in AIDS treatment and care. Traditional knowledge and practices, particularly from the great tradition of China and Ayurveda, will have an important role in bioprospecting and bring experiential wisdom to provide a safer and more cost-effective platform for drug discovery (Patwardhan and Gautam, 2005).

Rhus chinensis is native to China and Japan where it is known as Chinese Sumac. It has been used for treatment of cold, fever, cough and malaria. Moreover, aqueous extract from the gall of *Rhus chinensis* have activity on alpha-glucosidase (Shim et al., 2003). In the present study, the anti-HIV-1 activities of extracts from *Rhus chinensis* were investigated. We found that extracts from stem of *Rhus chinensis*, especially petroleum ether fraction (RC-1), suppressed significantly HIV-1 activity in vitro.

2. Materials and methods

2.1. Reagents and chemicals

AZT was purchased from Sigma. Horseradish peroxidase (HRP)-labeled goat anti-human IgG was purchased from Sino-America Biotechnology Company (China). p5F1, monoclonal antibody (McAb) against HIV-1 p24, was prepared by our laboratory. Human polyclonal anti-HIV-1 serum was kindly donated by Dr. Hiroo Hoshino (Gunma University School of Medicine, Japan).

2.2. Species collection

The whole plant of *Rhus chinensis* was collected in the Botanic garden, Kunming Institute of Botany, Chinese Academy of Sciences in May 2003. It was identified as *Rhus chinensis* Mill by Dr. Li-Gong Lei. A voucher of species (KUN 2004-04-56) is deposited in Kunming Institute of Botany.

2.3. Extract preparation

The powdered stems of *Rhus chinensis* Mill (200 g) was extracted under reflux (3 × 800 ml, 2 h for each time) with petroleum ether, ethyl acetate, butanol and water, respectively. The solvent was evaporated in vacuum to afford petroleum ether fraction (3.05 g, yield 1.75%); ethyl acetate fraction (RC-2; 3.0 g, yield 1.50%); butanol fraction (RC-3; 13.0 g, yield 6.0%); water fraction (RC-4; 1.5 g, yield 0.75%).

2.4. Cells and virus

Cell lines used in this study (C8166, H9 and HIV-1_{IIIB}/H9) were maintained in RPMI-1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco). The cells used in all experiments were in log-phase growth. HIV-1_{IIIB} was obtained from the culture supernatant of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was determined and calculated by the Reed and Muench method. Virus stocks were stored in small aliquots at −70 °C. The titer of virus stock was 3.4×10^6 $TCID_{50}$ per ml.

2.5. Cytotoxicity assay

The cellular toxicity of extracts on C8166 cells was assessed by MTT method as described previously (Zheng et al., 1995). Briefly, cells were seeded on a microtiter plate in the absence or presence of various concentrations of extracts in triplicate and incubated at 37 °C in a humidied atmosphere of 5% CO_2 for 72 h. The supernatants were discarded and MTT reagent (5 mg/ml in PBS) was added to each wells, then incubated for 4 h, 100 μl of 50% DMF–20% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 595/630 nm. The cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was calculated from dose–response curve.

2.6. Syncytium reduction assay

In the presence of 100 μl of various concentrations of extracts, C8166 cells (4×10^5 ml^{-1}) were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of 0.06. The final volume per well was 200 μl. AZT was used for drug control. After 3 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope. Percentage inhibition of syncytial cell formation was estimated from the percentage of syncytial cell number in treated culture to that in infected control culture and 50% effective concentration (EC_{50}) was calculated (Wang et al., 2004a).

2.7. ELISA for HIV-1 p24 antigen

The effect of extracts on HIV-1 replication in vitro was measured by antigen expression using capture ELISA as described previously (Zheng et al., 2000). Briefly, Triton X-100 treated cell-free culture medium was added to 96-well microtiter plates coated with McAb p5F1. The plates were then incubated with diluted human polyclonal anti-HIV-1 serums, followed by incubation with HRP-labeled goat anti-human IgG, and OPD substrate was added into the wells. The optical density of the plates was read on ELISA reader at 490/630 nm. The inhibition percentage of p24 antigen expression was calculated. The concentration of reducing p24 antigen expression by 50% (EC_{50}) was determined from the dose–response curve.

2.8. Protection for HIV-1 induced lytic effects

The activities of extracts against acute HIV-1 infection were based on the inhibition of HIV-1 induced cytopathogenicity in MT-4 cells as described previously (Pauwels et al., 1988). Mock-infected (uninfected) or HIV-1_{IIIB}-infected (MOI = 0.1) MT-4 cells (4×10^5 cells/ml) were seeded in 96-well flat-bottomed microtiter culture plates with 100 μl of different concentrations of extracts. AZT was used for control drug. After 7 days incubation at 37 °C, the viability of both HIV-1- and mock-infected cells were assessed by MTT method. The data are expressed as percentages of untreated uninfected control.

Table 1
Summaries of extracts of *Rhus chinensis* on anti-HIV-1 activities and cytotoxicities

Samples	Fractions	Cytotoxic (CC_{50}, μg/ml)	Syncytia (EC_{50}, μg/ml)	p24 antigen (EC_{50}, μg/ml)	RT activity (%)	Protection (%)	Co-culture (EC_{50}, μg/ml)
RC-1	Petroleum ether	71.6	0.7	0.9	−27.3 (100)	35.2 (40)	43.4
RC-2	Ethyl acetate	152.5	26.9	15.1	−26.6 (100)	NP	85.9
RC-3	Butanol	>200	17.4	21.9	11.4 (200)	NP	31.0
RC-4	Aqueous	>200	186.2	NI	−20.5 (200)	NP	NI

NI, no inhibition; NP, no protection. Values in parenthesis are given in μg/ml.

2.9. Inhibition assay of recombinant HIV-1 RT activity

HIV-1 RT activity was measured by ELISA RT kit using a commercially available kit (Roche) according to the instructions of the manufacturer. The extracts were incubated with DIG-labeled reaction mixture at 37 °C for 2 h, then anti-DIG-POD solution was added, followed by substrate ABTS. Foscarnet was used as a positive control. The absorbency at 405/490 nm ($A_{405/490}$) was read in the ELISA reader (Wang et al., 2004b).

2.10. Co-cultivation assay

3×10^4 C8166 cells co-cultured with 1×10^4 HIV-1_{IIIB} chronically infected H9 cells in the presence or absence of the extracts with various concentrations at 37 °C in a humidified atmosphere of 5% CO_2. Dextran sulfate (DS) was used as positive control. After 4 h incubation, the number of syncytia was scored under an inverted microscope (Wang et al., 2004c).

3. Results

3.1. Anti-HIV-1 activities of RC

The cytotoxicities and anti-HIV-1 activities of all the extracts from stem of *Rhus chinensis* (RC-1, RC-2, RC-3 and RC-4) are summarized in Table 1. The cytotoxicity of extracts on C8166 is shown in Fig. 1A. The RC-1, RC-2, RC-3 and RC-4 inhibited HIV-1 induced syncytium formation (Fig. 1B), and HIV-1

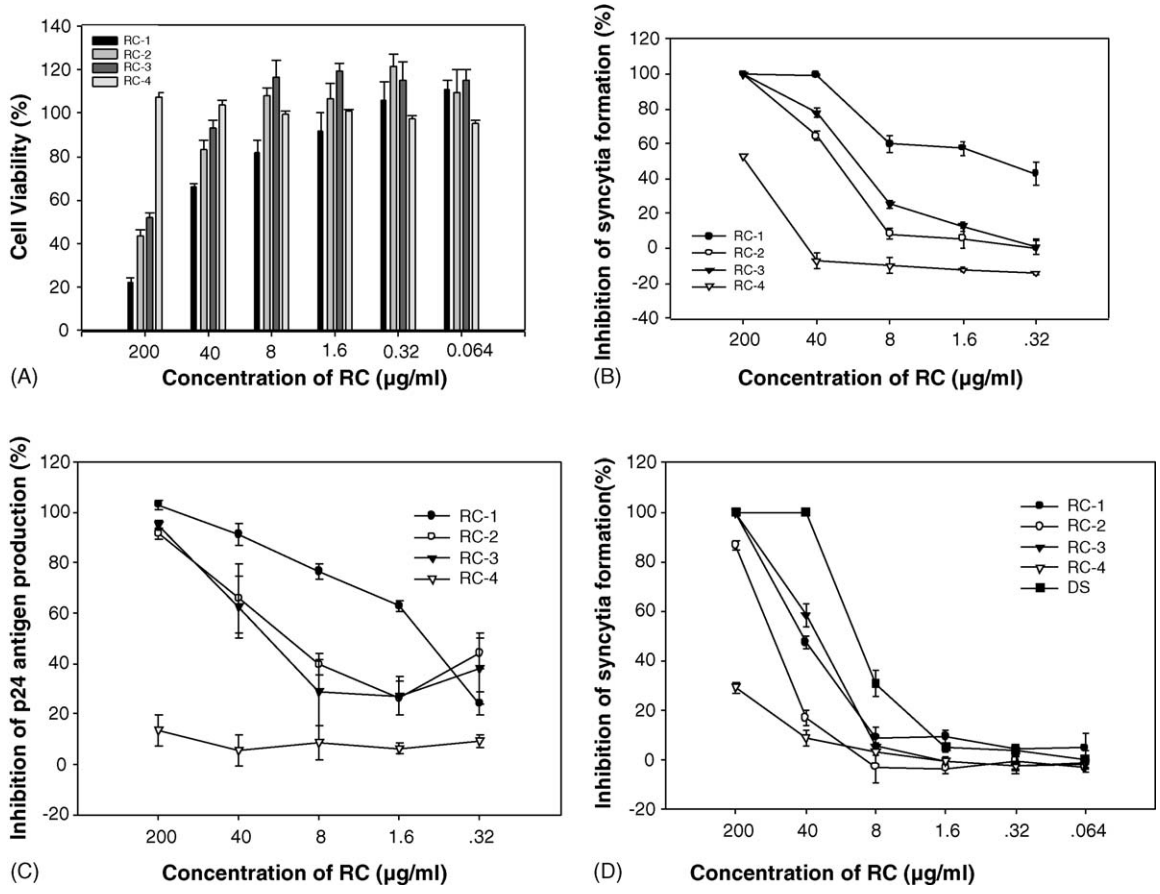

Fig. 1. Cytotoxicity and anti-HIV-1 activity of RCs. Cytotoxicity on C8166 cells was measured by MTT assay (A), inhibition of HIV-1 induced syncytium formation was quantified under inverted microscope (B), inhibition of HIV-1 p24 antigen in culture supernatants was performed by ELISA (C) and inhibition of fusion between normal C8166 cells and HIV-1_{IIIB} chronically infected H9 cells was quantified under inverted microscope (D). Data are expressed as means ± S.E.M. of at least three independent measurements.

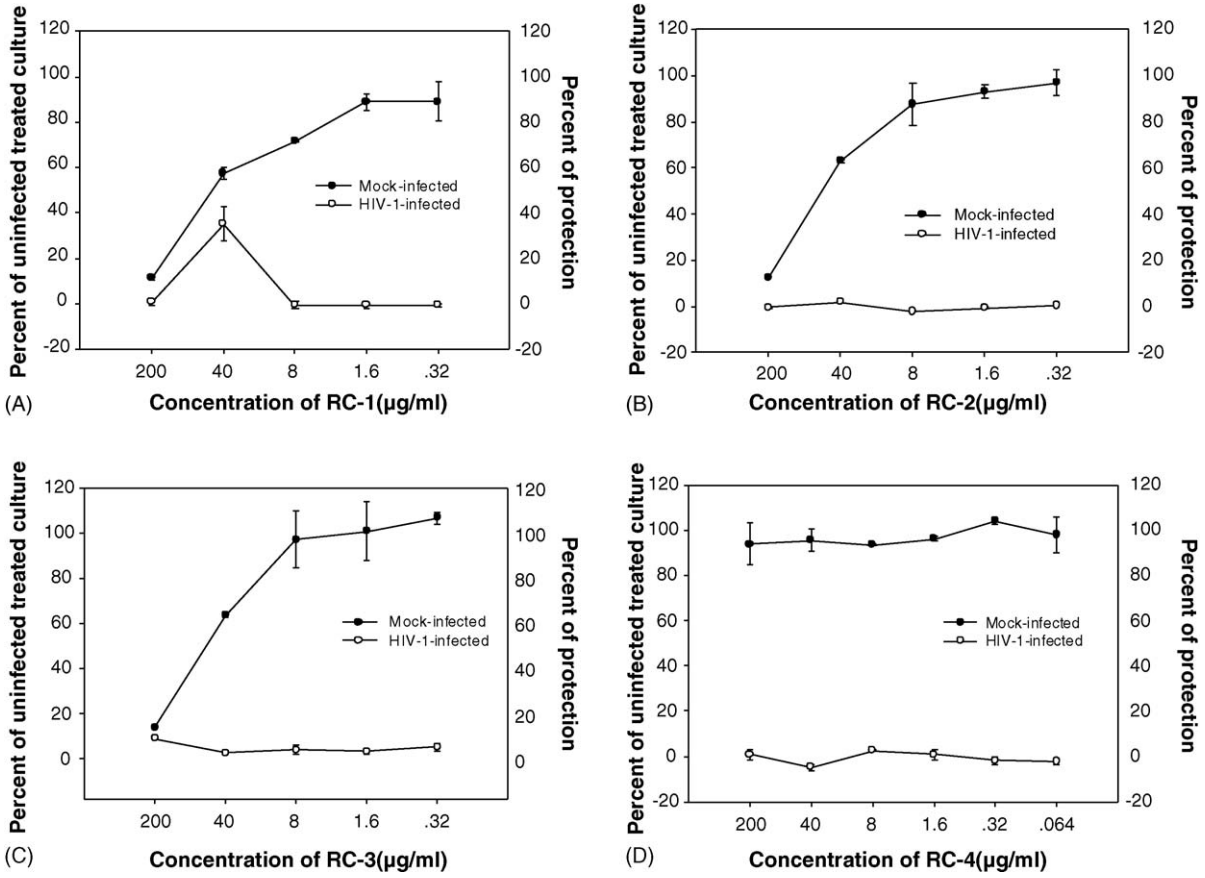

Fig. 2. (A–D) Protection of RCs on HIV-1 induced cell lytic effects. The data are expressed as percentages of untreated uninfected control.

p24 antigen (Fig. 1C). The EC_{50s} of RC-1 on inhibiting HIV-1 induced syncytium formation and p24 antigen were lower than those of RC-2, RC-3 and RC-4; RC-1, RC-2 and RC-3 showed slight ability on inhibiting the fusion between the normal C8166 and the HIV-1_{IIIB} chronically infected H9 cell in co-cultivation. RC-4 did not show this action (Fig. 1D).

3.2. Protection of RC on HIV-1-infected cells

Of all the extracts tested, only the RC-1 exhibited protection of HIV-1 induced cells lytic effects in vitro. At concentrations 40 μg/ml, the protection of infected cell was 35.2% (Fig. 2A), while the RC-2, RC-3 and RC-4 did not show this action (Fig. 2B–D).

3.3. Inhibition of RC on recombinant HIV-1 RT activity

RC-1, RC-2 and RC-4 did not inhibit the activity of recombinant HIV-1 RT. RC-3 showed slightly abilities on inactivating HIV-1 RT. The inhibition of RC-3 was 11.4% at concentration of 100 μg/ml (Table 1).

4. Discussion and conclusions

There has been a trend towards the use of traditional medicines to treat various diseases, especially in developing countries. Medicinal plants are chemical complex and diverse. They could provide a safer and more effective platform for newer scaffolds and could lead to better success than routine random screening. Botanical extracts provide a wide spectrum of biological and pharmacological properties, including cytoprotective, anticancer, anti-inflammatory, immunomodulative and anti-infectious activities (Patwardhan and Gautam, 2005). Many compounds with anti-HIV-1 effect have been screened out from botanicals and discovered to inhibit HIV at nearly all stages of viral life cycle. The anti-HIV activities of 15 medicinal plant extracts have been described (Bedoya et al., 2001).

In the present study, extracts of *Rhus chinensis* was shown to have anti-HIV activities, especially RC-1. The EC_{50s} of RC-1 (0.7 and 0.9 μg/ml) on inhibiting syncytium formation and p24 antigen were apparently lower than RC-2, RC-3 and RC-4. The CC_{50} of RC-1 on C8166 cell line was 71.6 μg/ml. The therapeutic index (TI) of RC-1 was about 100. RC-1 also had effect on protecting the infected cells of MT-4 cell line with the protection of 35.2%. These assays all show that RC-1 was effective against HIV-1.

Entry steps of HIV-1 might be one of the targets of RC-1. It inhibited the fusion between normal C8166 and HIV-1 chronically infected H9 cells in co-culture. In comparing the EC_{50} of inhibiting fusion, cytopathic effects and p24, the one for inhibiting fusion appeared to be much higher (Fig. 1D). This suggested that RC-1 had other targets on the HIV-1 replication

cycle. Since RC-1 did not inhibit the activity of recombinant HIV-1 RT (Table 1), other targets of RC-1 on HIV-1 replication may lie on the steps after reverse transcription. RC-2 and RC-3 also had anti-HIV-1 activity though the therapeutic index was much lower than RC-1. RC-3 demonstrated some inhibitory activity on HIV-1 RT (Table 1) and RC-4 was totally ineffective against HIV-1.

In conclusion, the extracts, especially the petroleum ether extract RC-1, of the medicinal plant *Rhus chinensis* was shown to have significant anti-HIV activity. The mechanism of anti-HIV-1 activity needs to be further studied and will be focused on the steps of HIV-1 after reverse transcription.

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