Anti-HIV-1 activities of extracts and phenolics from *Smilax china* L.

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Abstract: Four extracts (EtOH, $CHCl_3$, EtOAc, and BuOH) and five phenolics (dihydrokaempferol (1), resveratrol (2), kaempferol-7-O-β-D-glucoside (3), dihydrokaempferol-3-O-α-L-rhamnoside (4), oxyresveratrol (5)) from *Smilax china* L. was evaluated for anti-HIV-1 activities and cytotoxicity activities *in vitro*. All these extracts and phenolics showed lower or no cytotoxicity at a concentration ranged from 0.8 μg/mL to 100 μg/mL, but some showed potential anti-HIV-1 activities, that is, BuOH extract and compound 2 showed higher anti-HIV-1 activities than other extracts and compounds in the tested concentrations. EtOAc extract and compound 1 and 3 showed moderate anti-HIV-1 activities at a concentration higher than 4 μg/mL. In the end, the structure-activity relationship of four extracts and five phenolics was discussed.

Keywords: *Smilax china* L., phenolics, TZMB-L cells, anti-HIV-1, pseudotyped virus, structure-activity relationship.

INTRODUCTION

Currently there is no vaccine available for efficiently preventing HIV from infection. The treatments of HIV/AIDS patients have to more depend on the antiretroviral therapy. The highly active anti-retroviral treatment (HAART) indeed have expanded patient life-time and improve life qualities, but these chemosynthetic drugs can not eradicate the persistently or latently infected viruses, and easily induce drug-resistance; the strong side-effects and poor patient compliance are other obstacles for hindering the clinically trials of these chemosynthetic drugs. Searching for new candidates or lead compounds with higher antiviral efficiency while lower cytotoxicity appears essential for anti-HIV drug development.

Traditional Chinese medicines (TCM) provide rich resources for screening anti-HIV compounds, and hundreds of natural components have been isolated and proved antiviral activities. In ethnobotany *Smilax* genus (Smilacaceae family), some active components isolated from the species of *S. glabra*, *S. Kampestris* and *S. corbularia* have been reported to possess anti-HIV activities (Abdel-Malek *et al*., 1996; Chu *et al*., 2006; Tewtrakul *et al*., 2006). *S. china*, another species of *Smilax* genus, is a small vine widely distributed in southern China, which is also used as food in Chongqing, China (Meng *et al*., 2003). The roots and tubers of *S. china* are known as 'Ba Qia' (or 'Jin Gang Teng') and function by dispelling wind, promoting diuresis, detoxifying, and dissipating blood stasis effects (Tao, AD 450). This species was included in "the Chinese Pharmacopoeia" (Chinese Pharmacopoeia Committee, 2005). In China, they are used for the treatment of rheumatic arthritis, detoxification, lumbago, gout, and tumor and inflammatory diseases (State Administration of Traditional Chinese Medicine of People's Republic of China, 1999). Previous pharmacological investigations have indicated that this plant has antitumor (Li *et al*., 2007), antiinflammatory, and antinociceptive properties (Shu *et al*., 2006).

As a part of continued research on poly-active components from TCM, four extracts and five isolated phenolics from *S. china* were investigated to evaluate anti-HIV-1 activities *in vitro* using a pseudotyped virus-cell-based assay (Wu *et al*., 2010; Wungsintaweekul *et al*., 2011).

MATERIAL AND METHODS

Cells, extracts and phenolics from S. china, and other chemicals

TZMB-L and 293T cell lines were obtained from the Institute Pasteur of Shanghai (CAS). The four extracts and five phenolics were extracted and isolated from dried sliced tubers of *S. china* (Wu *et al*., 2010). The purity of five phenolics was analyzed on HPLC, namely dihydrokaempferol (1) 98.1%, resveratrol (2) 97.6%, kaempferol-7-O-β-D-glucoside (3) 98.3%, dihydrokaempferol-3-O-α-L-rhamnoside (4) 97.8%, oxyresveratrol (5) 96.9%; the chemical structures of these five phenolics are shown in fig. 1. Other chemicals, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from BioDev Company, sodium dodecyl sulfate (SDS), 3'-azido-3'-deoxythymidine (AZT), dimethyl sulfoxide (DMSO), and N, N'-dimethyl formamine (DMF) were bought from Sigma-Aldrich (St. Louis, MO).

Bioactive assay

Cell culture: 293T, TZMB-L and infected HIV-luc/NL4-3 TZMB-L cells were propagated in the DMEM medium (Gibco®, Shanghai, China), which included 10% hyclone,

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25mM HEPES, 4.5 g/L D-glucose, L-glutamine, 100,000 IU penicillin, and 100 mg streptomycin.

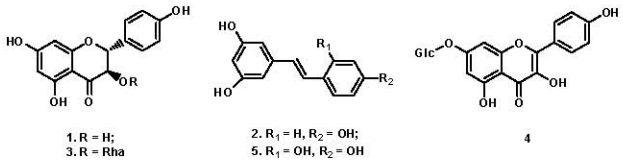

Fig. 1: Chemical structures of five phenolics from *S.china*

Pseudotyped virus construction and stocks

Pseudotyped HIV-luc/NL4-3 viruses (single-cycle infectious HIV-1 viruses) were generated by calcium phosphate co-transfection of HEK 293T cells with pLai-Δ-env-Luc and the expression plasmid for HIV-1 envelope protein (Env) of NL4-3 (X4-tropic), as previously described (Wang *et al.*, 2007). Virus stocks were quantified using $p24^{gag}$-capture enzyme-linked immunosorbent assay (ELISA).

Anti-HIV-1 assay

Next, the in *vitro* anti-HIV-1 activity of these four extracts and five phenolics were evaluated, and the well-established, TZBM-L-based detection system for viral infection was adopted. Hela-derived TZBM-L cells contain HIV-1-LTR (long term repeat)-derived luciferase gene, once the cells are infected, the virus-associated Tat protein can derive the luciferase expression, which activity can be measured by commercially available kit (Promega).

Cultured TZMB-L cells were infected by HIV-luc/NL4-3 (5 ng of $p24^{gag}$) for 2 h at 37°C in presence or not of tested candidates from *S. china* with different concentrations. Cells were then washed and cultured for an additional 3 days in presence or not of tested candidates. Viral infection was measured based on the detection of luciferase activity in cell lysates.

Cytotoxicity assay

In the end, the cell viability was also evaluated to make sure that the viral suppression was not due to the compound cytotoxicity. The cell viability was measured by MTT colorimetric assay, as previously described (Wang *et al.*, 2002). That is, TZMB-L cells (1×10^4 cells) were cultured in a 96-well plate with different concentrations of tested drugs, and the medium-treatment was used as a control. After incubation with drugs for 72 h in a 5% CO_2 incubator, the medium in each well was replaced with 20/μL of MTT (5 mg/mL, final concentration), and 4/h later, 100μL 10% SDS-50% DMF/well was added to dissolve the formed violet formazan crystals within the metabolically viable cells. The plates were incubated at 37°C overnight, then the OD values were read at 450/630 nm and cell viability was calculated.

RESULTS

Anti-HIV-1 effects of candidates from S. china

All the data were summarized in table 1 and fig. 2, AZT

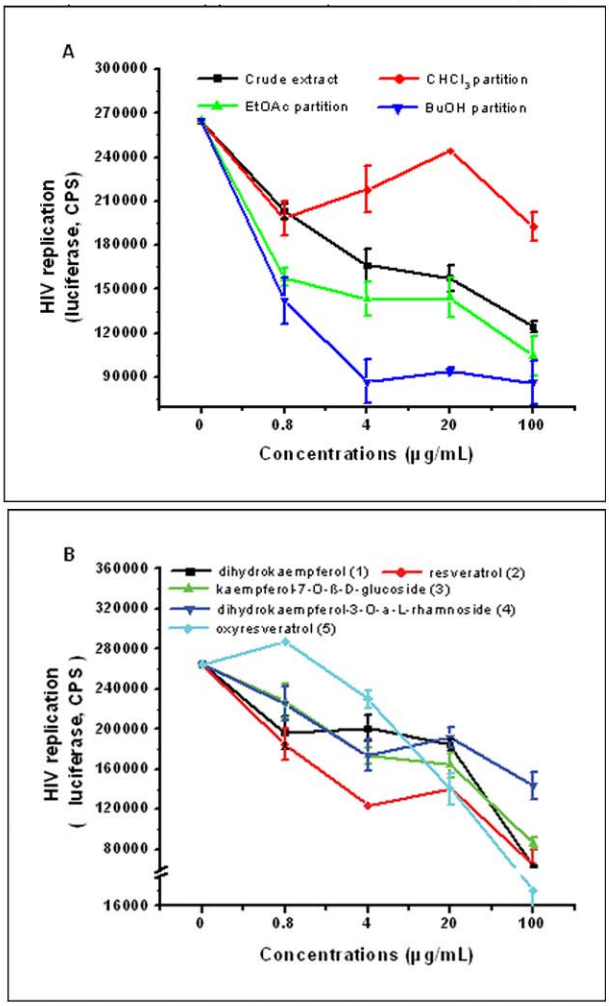

Fig. 2: The anti-HIV-1 effect of extracts and five phenolics from *S. china* on HIV-luc/NL4-3-infected TZMB-L cells

Cytotoxicity effects of candidates from S. china

All the data were summarized in fig. 3. Compound-mediated cytotoxicity should be considered prior to initiating an anti-HIV screening, the measurement of which is paramount for accurately determining antiviral activity (Blaira *et al.*, 2005).

DISCUSSION

As a positive control, AZT showed anti-HIV activity dramatically, and it's inhibition increased with the increase of AZT concentration. At 0.64 ng/mL, the inhibition rate was 87% (data not shown here). However, chronic, high-dose therapy with AZT is associated with significant side effects, including anemia, neutropenia (Fisher, 1997; 2003), hepatotoxicity (Takada *et al.*, 1993),

cardiomyopathy, and myopathy (Scruggs *et al.*, 2008), as well as damage to muscle cells, so there has been an increasing interest in alternative medicine and nontoxic therapeutic approaches to anti-HIV. It has been reported that natural components from the ethnobotany or diet are effective against HIV virus and are safe to the normal cell (Zhang *et al.*, 2008). In particular, flavonoids and stilbenoids, two types of compounds in a normal human diet and in many TCM, have been identified as beneficial agents in various disease states (Potapovich *et al.*, 2011), most common cancers (Kris-Etherton *et al.*, 2002; Mylonis *et al.*, 2010; Murthy *et al.*, 2012), cardiovascular disease (Kris-Etherton *et al.*, 2002; Mollace *et al.*, 2011), and neurodegenerative disorders (Ebrahimi *et al.*, 2012). So, it is necessary to find anti-HIV candidates with better efficacy but lower toxicity from natural resources.

As shown in table 1 and fig. 2 (A), in all tested concentrations, the anti-HIV-1 active rank of these extracts was: $CHCl_3$ < EtOH < EtOAc < BuOH. The inhibition rate of $CHCl_3$ extract less than 50% (the maximal inhibition rate was 27.32%±3.80%), showed no anti-HIV-1 activity; at a higher concentration (100 μg/mL), the EtOH crude extract and EtOAc showed 53.06%±1.31% and 60.65%±5.21% inhibition rates toward HIV-infected TZMB-L cells. BuOH extract showed more than 50% inhibition rates toward HIV-infected TZMB-L cells when the concentration was more than 4 μg/mL, especially at 100 μg/mL, the inhibition rate was 67.46%±5.65%; therefore BuOH extract showed the highest anti-HIV-1 activity among these four extracts.

In table 1 and fig. 2 (B), in lower concentrations (0-20 μg/mL), the anti-HIV-1 effect of compound 2 was the best, while compound 5 was lower among these five compounds in lower concentrations, surprisingly, at 100 μg/mL, compound 5 had higher activity (93.18±1.74%); Compound 1 and 3 showed moderate activity, and compounds 2 showed better activities. At 100 μg/mL, the inhibition rate of compounds 1, 2, and 3 toward HIV-infected TZMB-L cells was 76.30±0.34%, 75.84±6.09%, and 67.61±2.03% respectively, also showed better anti-HIV-1 activities. At 4 μg/mL, the inhibition rate of compound 2 was 53.22% higher than compounds 1 and 3. In general, compound 2 showed better anti-HIV-1 active compared to all these five compounds (table 1 and fig. 2). As shown in fig. 3, by the cytotoxicity measurement of extracts and five phenolics from *S. china* on TZMB-L cells, cells kept higher viability under the used drug concentrations. At the highest concentration of 100 μg/mL, more than 70% cells still could keep viable. So, these four extracts and five compounds showed lower or no cytotoxicity towards normal TZMB-L cells (fig. 3).

In the end, the structure-activity relationship of four extracts and five phenolics was discussed. Comparing the anti-HIV effects and cytotoxicity of these components with their physical chemical properties, we found that the anti-HIV effects of higher polar extract, BuOH, was higher than lower polar extracts, such as EtOAc and $CHCl_3$, such trend was the same as the cytotoxicity of these extracts even though the trend of cytotoxicity was not significant, so it is suggested that the anti-HIV effects of these extracts are related to polar property, this may be consistent with TCM decoction delivery in China (Zhang *et al.,* 2012). As for these five isolated phenolics, two parts were divided according to their structures, one is stilbene derivatives, the anti-HIV effect of resveratrol was higher than oxyresveratrol, and the cytotoxicity of resveratrol was also higher than oxyresveratrol accordingly even though the trend of cytotoxicity was not significant; Another is kaempferol derivatives, all the anti-HIV-1 effects were similar among all these three kaempferol derivatives even though the anti-HIV-1 activities of these three kaempferol derivatives were not reported before in pseudo-virus infected cells (Mahmood *et al.,* 1996; Likhitwitayawuid *et al.,* 2005).

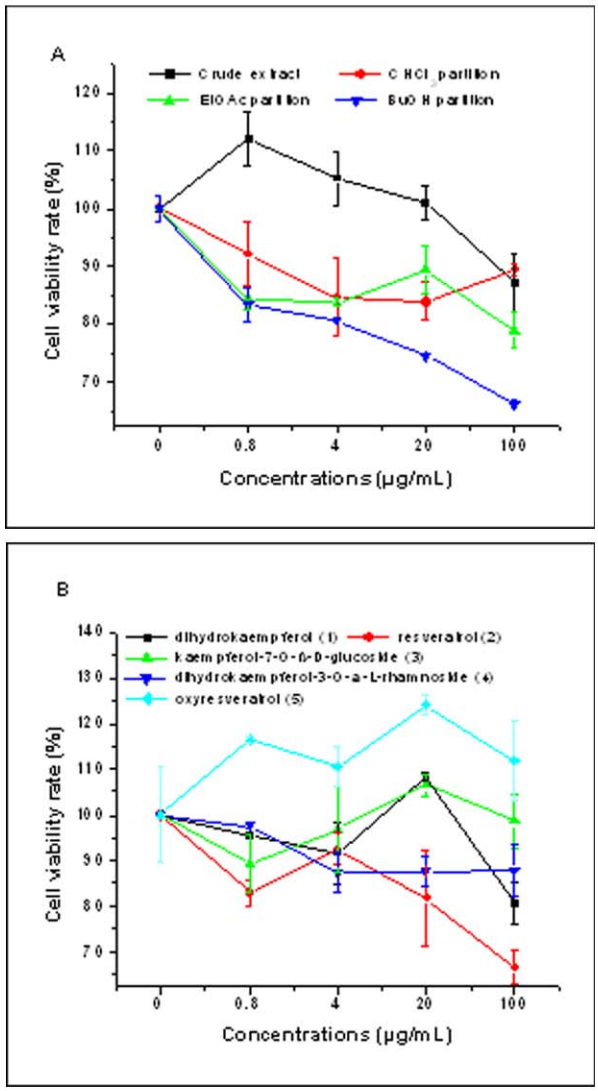

Fig. 3: The cytotoxicity of extracts and five phenolics from *S. china* on TZMB-L cells

Table 1: The inhibition rate of extracts and five phenolics from *S. china* on HIV-luc/NL4-3-infected TZMB-L cells

Concentrations (μg/mL)	Components				
	EtOH crude extract	$CHCl_3$ extract	EtOAc extract	BuOH extract	1
0	0	0	0	0	0
0.8	23.28±1.90*	25.13±4.37	40.38±2.28	46.28±6.00	25.79±6.02
4	37.24±4.36	17.70±5.86	45.93±4.48	67.18±5.67	24.15±4.73
20	40.58±3.20	7.78±0	45.51±5.13	64.35±0.63	30.50±2.17
100	53.06±1.31	27.32±3.80	60.65±5.21	67.46±5.65	76.30±0.34
Concentrations (μg/mL)	Components				
	2	3	4	5	
0	0	0	0	0	
0.8	30.09±5.85	13.88±6.39	14.97±6.41	0	
4	53.22±0	34.46±3.02	34.25±5.87	13.11±3.36	
20	46.81±0	38.00±4.57	27.72±4.01	46.99±5.99	
100	75.84±6.09	67.61±2.03	45.89±5.06	93.18±1.74	

1, 2, 3, 4, 5: dihydrokaempferol (1), resveratrol (2), kaempferol-7-O-β-D-glucoside (3), dihydrokaempferol-3-O-α-L-rhamnoside (4), oxyresveratrol (5); The inhibition rate here was expressed by percent (%).
* All the data are mean ± (S.D.) of triplicate tests.

All these extracts and phenolics showed lower or no cytotoxicity at a concentration ranged from 0.8 μg/mL to 100 μg/mL, but some showed potential anti-HIV-1 activities, that is, BuOH extract and compound 2 showed higher anti-HIV-1 activities than other extracts and compounds in the tested concentrations. EtOAc and compound 1, 3 showed moderate anti-HIV-1 activities at a concentration higher than 4 μg/mL.

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