Inhibition of HIV-1 Replication by Traditional Chinese Medicinal Herbal Extracts

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Abstract

Background: Highly active antiretroviral therapy (HAART) is the current treatment for HIV/AIDS and contains a combination of anti-HIV reverse transcriptase inhibitors and protease inhibitors. It is effective in suppressing HIV replication and subsequently improving the patients’ survival. However, the issues associated with use of HARRT such as the high cost, severe side effects, and drug resistance has called for development of alternative anti-HIV therapeutic strategies. In this study, we screened several traditional Chinese medicinal herbal extracts for their anti-HIV activities and determined their anti-HIV mechanisms.

Materials and Methods: Nine traditional Chinese medicinal (TCM) herbal plants and their respective parts derived from Hainan Island, China were extracted using a series of organic solvents, vacuum dried, and dissolved in dimethyl sulfoxide. Initial anti-HIV activity and cytotoxicity of these extracts were evaluated in HIV-infected human CD4⁺ T lymphocytes Jurkat. Extracts of higher anti-HIV activities and lower cytotoxicity were selected from the initial screening, and further examined for their effects on HIV-1 entry, post-entry, reverse transcriptase, gene transcription and expression using a series of virology and biochemistry strategies.

Results: Four extracts derived from two different herbal plants completely blocked HIV-1 replication and showed little cytotoxicity at a concentration of 10 µg/ml. None of these four extracts had any inhibitory effects on HIV-1 long terminal repeat promoter. Two of them exhibited direct inhibitory activity against HIV-1 reverse transcriptase (RT). All four extracts showed significant blocking of HIV-1 entry into target cells.

Conclusions: These results demonstrated that four TCM extracts were capable of preventing HIV-1 infection and replication by blocking viral entry and/or directly inhibiting the RT activity. These results suggest the possibility of developing these extracts as potential anti-HIV therapeutic agents.

Keywords: TCM; Anti-HIV; Entry inhibitors; RT inhibitors; Hainan; Tropical medicinal plants

Introduction

Acquired immune deficiency syndrome (AIDS) is caused by infection of human immunodeficiency virus type I (HIV-1) and is one of the most destructive pandemics [1]. The virus is presented in the form of both free viruses and virus-infected immune cells. HIV is a Lentivirus and mainly infects dendritic cells, CD4⁺ T lymphocytes, and monocytes/macrophages in the human immune system [2]. HIV-1 enters its target cells through interaction of viral glycoprotein spikes with CD4 and chemokine receptor CCR5 or CXCR4 on the cell surface. CCR5 is used by macrophage-tropic HIV-1 isolates at the early stage of HIV-1 infection, while CXCR4 is used by T cell-tropic HIV-1 isolates at later stages of HIV-1 infection [3-5]. Then, the viral envelope is fused with the cell membrane; the virion core including RNA genome, capsid, and viral enzymes is released into the cell. After the virion core enters the cell, reverse transcriptase converts single-stranded viral RNA into proviral DNA. This process often gives rise to mutations that offer drug-resistance or allow the virus to evade the body’s immune response because of the highly error-prone nature of reverse transcriptase. Proviral DNA is then imported into the nucleus and becomes integrated into host genome by viral integrase [6,7]. Viral RNA are transcribed from the integrated proviral DNA and exported into cytoplasm for viral protein synthesis [8,9]. The final step of the viral cycle is assembly of new HIV-1 virions, which occurs at the plasma membrane of the host cells. During the maturation of virions, HIV protease cleaves the polyproteins into individual functional HIV structural proteins and enzymes and the virus becomes mature and ready for the new round of infection [10].
The anti-HIV therapeutic strategy has been mainly targeted at RT and PR. RT inhibitors block the virus from synthesizing DNA from its RNA; PR inhibitors block cleavage of HIV polyproteins into individual viral structural proteins during virus maturation. Some advances have been recently made to target the viral entry and IN. Fusion inhibitors, the new class of drugs, block viral envelope fusion with cell membrane. IN inhibitors prevent proviral DNA from being integrated into host chromosome. The Food and Drug Administration (FDA) of the United States has approved a total of 22 anti-HIV-1 drugs, a majority of these drugs are HIV-1 RT and PR inhibitors [11]. Enfuvirtide are the two approved HIV fusion inhibitors. Maraviroc binds to CCR5, preventing an interaction with gp120 [12], while Enfuvirtide binds to gp41 and interferes with its ability to approximate the two membranes [13].

The current treatment for HIV infection is called highly active antiretroviral therapy (HAART) and is a combination of selected above-mentioned inhibitors. HAART is very effective in suppressing HIV-1 replication, but it cannot eradicate the virus from the infected subjects [14]. This therapy cannot achieve the consistent optimal results, because of side effects and the non-adherence issues. On the other hand, traditional Chinese medicine (TCM) constitutes an important form of medical care for various human diseases in East Asian countries for many thousands of years, and Chinese herbs-based medication is the most important part of TCM [15]. Unlike one single compound-based Western medicine, TCM is a mix of multiple ingredients that are carefully balanced and standardized to maximize their therapeutic efficacy while minimize their toxicity and side effects. Compared to HAART, TCM has fewer side effects, little drug resistance, and inexpensive. In some cases, TCM may be even used to lessen some side effects that are caused by HAART. Currently, HIV-1-inhibitory TCMs are reported to include Scutellaria baicalensis Georgi, Prunella vulgaris, Paeonia Suffruticosa, Rhizoma Polygoni Cuspidati, Radix Notoginseng, Ramulus Visci, and Ajuga Decumbens Thumb [16-21]. Our recent studies have added Euphorbiaceae, Trigonostemma xyphophylloides (TXE) and Dipterocarpaceae, Vatica astrotricha (VAD) onto this soon-to-be-rapidly-expanding list [22]. Further investigation is under way to fractionate these extracts and to define the active anti-HIV-1 entry constituents in these extracts including better characterization of their effects on the interaction between HIV-1 gp120 and CD4/chomine receptors CCR5 and CXCR4 [22].

In the current study, we continued to screen and characterize nine more extracts from Chinese medicinal herbal plants or their parts: leaves, stems and roots of Annonaceae Artabotrys pitosus (AAP), Annonaceae Oncodostigma hainanense (AOH) and Annonaceae Dasymaschalan rostratum (ADR), for their anti-HIV activities. These extracts have been used as TCM to treat malaria, cancers and bacterial and fungal infections, stop bleeding, heal fractures, and ameliorate bruises [23]. We also performed a series of studies to determine the underlying anti-HIV mechanisms of those extracts.

### Materials and Methods

#### Preparation of plant extracts

All plants used in this study were collected at the tropical national forest park Bawangling, Hainan Island, PR China (Table 1). Scientific names and classification of these plants were validated by the research team at Hainan Normal University, Haikou, PR China. Samples of these plants were kept at the Hainan Provincial Key Laboratory of Tropical Medicinal Herbal Chemistry, Haikou, PR China. Plant samples were air dried, ground and continued to be dried in a pressurized oven at 40°C and 0.08 MPa. The dried and ground materials were then subjected to three rounds of refluxing extraction in 75% ethanol at 80°C. The ethanol extracts were then concentrated to become ointment in a revolving depressurized vacuum evaporator at 55°C. The ointment was further lyophilized to the final form of powder and stored at desiccators. The powders were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and used as stock at -20°C.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Plant (family, species)</th>
<th>Sampling location</th>
<th>Sampling parts</th>
<th>Medicinal usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Annonaceae, Artabotrys pitosus Merr. et Chun</td>
<td>Bawangling</td>
<td>Leaves</td>
<td>Anti-malaria, TB</td>
</tr>
<tr>
<td>2</td>
<td>Annonaceae, Artabotrys pitosus Merr. et Chun</td>
<td>Bawangling</td>
<td>Stems</td>
<td>Anti-malaria, TB</td>
</tr>
<tr>
<td>3</td>
<td>Annonaceae, Artabotrys pitosus Merr. et Chun</td>
<td>Bawangling</td>
<td>Roots</td>
<td>Anti-malaria, TB</td>
</tr>
<tr>
<td>4</td>
<td>Annonaceae, Oncodostigma hainanense (Merr.) Tsiang et P.T.Li</td>
<td>Bawangling</td>
<td>Fruit</td>
<td>Bleeding, fractures, bruises</td>
</tr>
<tr>
<td>5</td>
<td>Annonaceae, Oncodostigma hainanense (Merr.) Tsiang et P.T.Li</td>
<td>Bawangling</td>
<td>Leaves</td>
<td>Bleeding, fractures, bruises</td>
</tr>
<tr>
<td>6</td>
<td>Annonaceae, Oncodostigma hainanense (Merr.) Tsiang et P.T.Li</td>
<td>Bawangling</td>
<td>Stems</td>
<td>Bleeding, fractures, bruises</td>
</tr>
<tr>
<td>7</td>
<td>Annonaceae, Dasymaschalan rostratum Merr. et Chun</td>
<td>Bawangling</td>
<td>Roots</td>
<td>Anti-tumor</td>
</tr>
<tr>
<td>8</td>
<td>Annonaceae, Dasymaschalan rostratum Merr. et Chun</td>
<td>Bawangling</td>
<td>Stems</td>
<td>Anti-tumor</td>
</tr>
<tr>
<td>9</td>
<td>Annonaceae, Dasymaschalan rostratum Merr. et Chun</td>
<td>Bawangling</td>
<td>Leaves</td>
<td>Anti-tumor</td>
</tr>
</tbody>
</table>

**Table 1:** Nine Chinese herbal medicinal plants and parts and their medicinal usage.

### Cells and chemicals

Jurkat and CEM-GFP cells stably expressing HIV-1 long terminal repeat promoter-driven green fluorescence protein (GFP) were cultured in RPMI-1640 medium (Lonza, Wwalkersville, MO) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and 100 units/ml penicillin (Sigma, St. Louis, MO). G418 (Lonza) was included in the medium (1.25 mg/ml) for CEM-GFP cells. U87.CD4.CXCR4 and U87.CD4.CCR5 cells stably expressing CD4/ CXCR4 and CD4/CCR5 respectively were obtained from the NIH...
AIDS Reagent Program and cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin.

Preparation and infection of HIV-1 pseudotyped viruses HXB2 and YU-2

293T cells were plated in a 100 mm dish at a density of 2 x 10^6 cells per plate and transfected with 20 μg HIV-Luc plasmid and 4 μg pHXB2-env, pYU2-env, pVSV-G, or pcDNA3 by the calcium phosphate precipitation method [22]. Forty-eight hours post transfection, the cell culture supernatant was collected, cleared of cell debris, and used for infection. The virus titers were determined by reverse transcriptase assay [24] and expressed as counts per min per milliliter (cpm/ml). For infection, pseudotyped viruses corresponding to a 2,000 cpm RT activity were used to infect target cells. Two hours post infection, the culture medium was replaced with the fresh DMEM, the cells were continued to incubate for 48 hr and harvested for the Luc activity assay [25,26].

HIV replication assay

One million Jurkat were infected with HIV-1 corresponding to a 10,000 cpm RT activity in a total volume of 1 ml. Twenty-four hours post infection, cells were treated with plant extracts at indicated concentrations or an equivalent concentration of the DMSO solvent. The culture supernatants were collected for the RT activity assay every other day, while fresh extracts or DMSO was replenished.

Cytotoxicity assay

The cytotoxicity of these extracts was determined by using the trypan blue exclusion method. Briefly, aliquots of HIV-infected and extracts-treated Jurkat were collected every other day, stained in 0.2% trypan blue dye, then counted for viable cells under a light microscope. Uninfected and HIV-infected and DMSO-treated Jurkat were collected every other day, stained in 0.2% trypan blue dye, then counted for viable cells under a light microscope. Uninfected and HIV-infected and DMSO-treated Jurkat were included as controls.

Flow cytometry analysis

CEM-GFP cells were treated with 10 μg/ml each of the extracts for 3 days or 7 days, and then harvested to determine the GFP expression by flow cytometry.

Data analysis

Results were expressed as mean ± SEM of triplicates and representative of multiple independent experiments. The statistical significance of the differences between the means of the experimental groups was tested by two-tailed student’s t-test. A difference was considered as significant when p is <0.05, and highly significant when p is <0.01.

Results

Anti-HIV activity of the extracts of traditional Chinese medicinal herbal plants

To determine whether any of the nine selected extracts (Table 1) have anti-HIV activity, we infected CD4+ T lymphocytes Jurkat with a replication-competent T-tropic HIV-1 strain HXB2, and then treated the cells with these extracts at concentrations of 10 μg/ml or 100 μg/ml for 2 weeks. DMSO was included as a vehicle control. Mock-infected Jurkat and HIV-infected cells without any treatments were also included as controls. Compared to the DMSO control, there were low HIV-1 replications in cells that were treated with 10 μg/ml extracts from the leaves, stems and roots of Annonaceae, Artabotrys pilosus (AAP-leaves, stems, and roots) and from the roots of Annonaceae Dasymaschalon rostratum (ADR-roots) (Figure 3A). However, the other five extracts did not show any effects on HIV-1 replication (data not shown). Throughout the above infection experiments, we also monitored cell survival of all treatments by trypan blue staining. At the concentration of 10 μg/ml, the cell viability in all extract treatments was similar to that in mock or DMSO-treated cells (Figure 2). Nevertheless, at a concentration of 100 μg/ml, the cell viability in all DMSO-treated and extracts-treated cells began to decline at day 5 (data not shown). Based on these findings, the extract concentration of 10 μg/ml was chosen for all following mechanistic studies.

Inhibition of HIV-1 replication by these extracts at lower concentrations

We then determined whether the inhibitory effects on HIV-1 replication of these extracts could be achieved at lower concentrations. Similarly, we infected Jurkat with HIV-1 viruses and then treated the cells with each of the extracts at a concentration of 1 μg/ml or 5 μg/ml. Fresh extracts were added every other day, while the supernatants were collected for the RT activity assay. At the concentration of 1 μg/ml, the extracts only showed inhibition of HIV-1 replication at day 8 and 10 post infection compared to the DMSO control (Figure 3A). At the concentration of 5 μg/ml, these extracts showed some inhibition at day 6 post infection and more inhibition at day 8 and 10 post infection (Figure 3B). At both 1 μg/ml and 5 μg/ml concentrations, extracts did not show any effects on cell viability (data not shown). These results

<table>
<thead>
<tr>
<th>Extract Type</th>
<th>RT Activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP-leaves</td>
<td>75000</td>
</tr>
<tr>
<td>AAP-stems</td>
<td>60000</td>
</tr>
<tr>
<td>AAP-roots</td>
<td>45000</td>
</tr>
<tr>
<td>ADR-leaves</td>
<td>30000</td>
</tr>
<tr>
<td>ADR-stems</td>
<td>15000</td>
</tr>
<tr>
<td>ADR-roots</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>75000</td>
</tr>
<tr>
<td>W/O</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1: Effects of extracts on HIV-1 replication. One million Jurkat were infected with HIV-1 HXB2 corresponding to a 10,000 cpm RT activity for 24 hr and then treated with 10 μg/ml fresh extracts from the leaves, stems and roots of Annonaceae, Artabotrys pilosus (ARP-leaves, stems, and roots) and the roots of Annonaceae, Dasymaschalon rostratum (ADR-roots) every other day. The cell culture supernatants were collected at indicated time points for the RT activity assay. All extracts were dissolved in DMSO, and DMSO was used as a vehicle control. Mock-infected Jurkat cells (Uninfected) and HIV-infected cells without any treatments (W/O) were also included as controls. These data were representative of three independent experiments.
suggest a seemingly dose-dependent inhibition of HIV-1 replication by these extracts and also support our choice of the 10 μg/ml concentration of these extracts throughout our remaining studies.

Block of HIV-1 entry by these extracts

HIV-1 infection begins with HIV-1 envelope gp120 binding to CD4 and chemokine co-receptors CCR5 for M-tropic HIV-1 stains or CXCR4 for T-tropic HIV-1 stains. We then determined whether these extracts would block viral entry. We took advantage of the replication-defective single round HIV-Luc reporter system [27]. In this reporter system, HIV-1 env gene is nonfunctional and the firefly luciferase gene is inserted into HIV-1 nef. The reporter virus system allows in trans complementation of env genes to not only study HIV-1 tropism, but also accurately monitor HIV-1 entry. To this end, we transfected HIV-Luc reporter and T-tropic HIV-1 HXB2 or M-tropic HIV-1 YU-2 envelope DNA and obtained HXB2 or YU-2 envelope-pseudotyped HIV-Luc viruses. We pre-treated U87.CD4.CXCR4 or U87.CD4.CCR5 cells with 10 μg/ml each of extracts for 1 hr, then infected these cells with the pseudotyped viruses (HXB2 for U87.CD4.CXCR4; YU-2 for U87.CD4.CCR5). We also included pseudotyped viruses without any envelope and vascular stomatitis virus envelope glycoprotein (VSV-G)-pseudotyped viruses as negative and positive controls, respectively. Compared to the DMSO treatment, all extract treatments showed significantly lower levels of the luciferase activity in HXB2-infected U87.CD4.CXCR4 cells (Figure 4A) or YU-2-infected U87.CD4.CCR5 cells (Figure 4B). These extracts showed little effects on entry of VSV-G-pseudotyped viruses, while HIV-Luc pseudotyped without any envelopes had no luciferase assay readings (data not shown). These results indicate that pre-treatment of these extracts completely blocked entry and infection of both T-tropic and M-tropic HIV viruses.

No effects of the extracts on HIV-1 infectivity

We next determined whether these extracts would have any direct effects on HIV-1 infectivity. To this end, we directly incubated HXB2- or YU-2-pseudotyped HIV-Luc viruses with the extracts at 37°C for 2 hr. We then recovered the viruses by centrifugation and infected U87.CD4.CXCR4 or U87.CD4.CCR5 cells with these viruses. Compared to the DMSO control, none of the extract treatments showed significant differences of the luciferase activity (Figure 5), suggesting that these extracts are not able to inactivate HIV-1.
Figure 5: Effects of extracts on HIV-1 infectivity. HIV-Luc virus pseudotyped with HXB2 (A) or YU-2 envelope (B) was first incubated with 10 µg/ml each of the extracts at 37°C for 2 hr. The virus was recovered by centrifugation and then used to infect either U87.CD4.CXCR4 (for HXB2) or U87.CD4.CCR5 cells (for YU-2). The cells were harvested for the luciferase reporter gene assay 48 hr post infection. The data were mean ± SEM of triplicate experiments and representative of three independent experiments.

Figure 6: Effects of extracts on HIV-1 post-entry. U87.CD4.CXCR4 and U87.CD4.CCR5 cells were first infected with HIV-Luc virus pseudotyped with HXB2 (A) or YU-2 envelope (B). Following medium change, the cells were treated with 10 µg/ml each of the extracts for 48 hr and then harvested for the luciferase reporter gene assay. The data were mean ± SEM of triplicate experiments and representative of three independent experiments.

Effects of the extracts on HIV-1 post-entry

To determine whether these extracts would have any inhibitory effects on any post-entry events of HIV life cycle, we infected U87.CD4.CXCR4 with HXB2-pseudotyped HIV-Luc or U87.CD4.CCR5 cells with YU-2-pseudotyped HIV-Luc, washed off the unbound viruses, and then treated the cells with the extracts. Compared to the DMSO control, only extracts from AAP-leaves and AAP-stems showed significant changes in the luciferase activity (Figure 6), suggesting that these two extracts have inhibitory effects on the post-entry events of HIV life cycle.

Inhibition of HIV-1 RT activity by extract of AAP-leaves and AAP-stems

Reverse transcriptase (RT) is absolutely required for the HIV-1 life cycle, as it converts the single-stranded RNA genome to a DNA molecule. Thus, we next determined whether these two extracts would have any direct inhibitory effects on HIV-1 RT enzymatic activity. To this end, we purified the HIV-1 virions, lysed them to release the RT, and then determined the RT activity in the presence of the extracts. We also included 0.1% DMSO as a vehicle control and AZT as a positive control in these experiments. Compared to AZT treatment, extracts of AAP-leaves and AAP-stems but not AAP-roots and ADR-roots significantly inhibited HIV-1 RT enzymatic activity (Figure 7).

Figure 7: Effects of extracts on the HIV-1 RT activity. Purified HIV-1 virions were incubated with 10 µg/ml each of the extracts for the RT activity assay. The RT inhibitor AZT (5 µM) was included as a positive control, while DMSO was used as the vehicle control for the extracts. The data were mean ± SEM of triplicate experiments and representative of three independent experiments.

No inhibition of HIV-1 LTR promoter activity by the extracts

The HIV-1 long terminal repeat (LTR) promoter controls HIV-1 gene transcription and is an important regulatory step of HIV-1 gene expression. Thus, we also determined whether these extracts would affect the HIV-1 LTR promoter-driven transcription. We took advantage of the CEM-GFP cells in which the green fluorescence protein (GFP) is under the control of the HIV-1 LTR promoter [28]. We treated these cells with 10 µg/ml of each extract for 3 days or 7 days and determined the relative GFP expression level. We treated the cells with extracts for 7 days to ensure complete GFP turnover. The results showed no significant difference of GFP intensity between each of the extracts and the DMSO control (Figure 8), suggesting that the extracts have no inhibitory effects on HIV-1 LTR promoter activity and gene transcription.

Discussion

In this study, we screened nine new TCM herbal extracts from Hainan Island, China for their anti-HIV-1 effects (Table 1). These extracts are from leaves, stems and roots of three different Chinese medicinal herbal plants from Annonaceae family-AAP, AOH and ADR. Annonaceae family, which consists of 2300 to 2500 species and more than 130 genera, their stems, leaves and roots of some species are used as Chinese traditional medicine. Some chemical constituents of the leaves and stems from these species are well known for their antifungal, bacteriostatic, antimalarial, and cytostatic effects. The chemical compounds, including flavonoids (FL), alkaloids (AL) and acetogenins (AT), have been extracted [29]. FL possesses anti-inflammatory, antithrombotic, ant carcino genic, and antiviral incudling anti-HIV-1 activities [30]. The anti-HIV-1 activity of FL compounds been implicated in the inhibition of RT enzyme [31]. Baicalin (BA, 7-
glucuronic acid, 5,6-dihydroxyflavone) is purified from FL compound that have also been known to possess anti-inflammatory and anti-HIV-1 activities through strengthening host defense. In comparison to FL compounds, BA has been found to inhibit HIV-1 infection of human peripheral blood mononuclear cells by interfering with viral entry, through the interaction between HIV-1 envelope proteins and the cellular CD4 and chemokine receptors (both CD4/CXCR4 or CD4/CCR5) [32-34].

![Figure 8: Effects of extracts on the HIV-1 long terminal repeat (LTR) promoter activity. CD4+ T lymphocytes CEM stably expressing green fluorescence protein (GFP) under the control of HIV-1 LTR promoter were treated with 10 µg/ml each of the extracts for 3 days (A) and 7 days (B). Then, the cells were collected for GFP expression by flow cytometry. The data were the geometric means of the GFP expression level and were mean ± SEM of triplicate experiments and representative of three independent experiments.](image)

In the study, we showed that extracts from AAP-leaves, AAP-stems, AAP-roots and ADR-roots completely blocked HIV-1 replication at concentration of 10 µg/ml (Figure 1) without apparent cytotoxicity (Figure 2). Lower concentrations of those extracts were also tested and found to be HIV-inhibitory as well albeit to less extent (Figure 3). We then attempted to determine the anti-HIV mechanisms by examining their effects on viral entry, infectivity, post-entry, RT, and gene transcription. We showed that the extracts inhibited viral entry (Figure 4), but had no effects on viral infectivity (Figure 5) and gene transcription (Figure 8). We also showed that extracts from AAP-leaves and AAP-stems exhibited HIV inhibitory effects at post-entry steps (Figure 6), likely through its inhibitory effects on HIV RT enzymatic activity (Figure 7). Taken together, these results indicate that extracts from AAP-leaves, AAP-roots, AAP-stems and ADR-roots all block HIV-1 replication by interfering with HIV-1 entry, and that AAP-leaves and AAP-stems also block HIV-1 replication by interfering with HIV-1 RT enzymatic activities. These results are consistent with the previous findings that chemical compound FL inhibits HIV-1 infection at the level of viral entry and RT enzymatic activity [34]. Further studies are clearly needed to determine whether these extracts inhibited T cell tropic (X4) and macrophage tropic (R5) HIV-1 Env protein-mediated fusion with CD4/CXCR4 or CD4/CCR5-expressing cells, or inhibited binding of HIV-1 gp120 to CD4, and how AAP-leaves and AAP-roots inhibited HIV-1 RT enzymatic activities. In addition, it also merits further investigation whether those HIV-inhibitory extracts have effects on expression of HIV receptors (CD4, CCR5, and CXCR4), or β-chemokines on target cells.

With the history of several thousand years, TCM has been used to treat various illnesses. There are only a few of Chinese medicinal herbs available for treating HIV/AIDS patients: Immunity-boosting herbs, such as astragalus, echinacea, and ginkgo are used to help strengthen an ailing immune system; and certain herbs such as garlic are used to battle bacteria and viruses. Deglycyrrhizinated licorice is shown to soothe the mouth and throat ulcers that often are often detected in full-blown AIDS patients. In this study, we found four potential extracts from Annonaceae Family: AAP-leaves, AAP-stems, AAP-roots and ADR-roots, with anti-HIV-1 activity. It is clear that further studies including clinical trials on these Chinese medicinal herbs for treatment of HIV/AIDS are warranted.

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**References**


