

EFFECTS OF TRADITIONAL CHINESE MEDICINAL
HERBAL EXTRACTS ON HIV-1 REPLICATION

Ting Wang

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Johnny J. He, Ph.D. Committee Chair

Andy Yu, Ph.D.

Master's Thesis
Committee

Robert H. Schloemer, Ph.D.

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ABSTRACT

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Background: The current treatment for HIV/AIDS is called highly active antiretroviral therapy (HAART) and is a combination of anti-HIV reverse transcriptase inhibitors and protease inhibitors. HAART is capable of 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 have 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.

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 combined virology, cell biology and biochemistry techniques.

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.

Johnny J. He, Ph.D. Committee Chair

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LIST OF ABBREVIATIONS

AAP	<i>Annonaceae, Artabotrys pilosus</i>
ADR	<i>Annonaceae, Dasymaschalon rostratum</i>
AOH	<i>Annonaceae, Oncodostigma hainanense</i>
TCM	traditional Chinese medicine
HIV-Luc	a HIV-1-based reporter virus containing inactive <i>nef</i> and <i>env</i> genes and having the luciferase (Luc) gene inserted at the 5' end <i>nef</i> gene
HXB2-env	envelope from HIV-1 isolate HXB2
YU-2-env	envelope from HIV-1 isolate YU-2
VSV-G	vascular stomatitis virus envelope glycoprotein
RT	reverse transcriptase
DMSO	dimethyl sulfoxide
AZT	azidothymidine

INTRODUCTION

HIV/AIDS epidemics

Acquired immune deficiency syndrome (AIDS) is caused by infection of human immunodeficiency virus type 1 (HIV-1) and is one of the most destructive pandemics [1]. It has killed more than 25 million people since it was first diagnosed in 1981. Despite improved access to antiretroviral treatment and improved care in many regions of the world, the AIDS pandemic claimed an estimated 2.8 million (between 2.4 and 3.3 million) lives in 2005, of which more than half a million (570,000) were children [2, 3]. Sub-Saharan Africa is the worst-affected region and has an estimated 21.6 to 27.4 million people currently living with HIV [4, 5]. The epidemic is spreading most rapidly in Eastern Europe and central Asia, where the number of people living with HIV increased 67% between 2001 and 2008 [6, 7].

Based on the epidemic data in 2008, about half of the 31.3 million HIV/AIDS population are women and about 98 percent of these infected women are in developing countries [8]. These figures suggest that women had a greater risk of acquiring HIV through heterosexual intercourse. Studies have shown that women are twice more likely to become infected with HIV through unprotected heterosexual intercourse than men [9]. There are a large number of HIV-infected women and women with AIDS. As a result of vertical transmission from HIV/AIDS mothers to their infants during pregnancy, birth or breastfeeding, more and more children are living and affected by HIV/AIDS. In Africa, one in every three newborn children is infected with HIV and dies before the age of one, and most of these infected children have died before they are five years old.

HIV transmissions and symptoms

HIV can be transmitted via bodily fluids, such as blood, semen, vaginal fluid, pre-ejaculate, or breast milk. 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 [10]. Infection with HIV-1 is associated with a decrease in CD4⁺ T cell count and an increase in viral load. HIV infection can be divided into four different stages based on the patient's CD4⁺ T cell count, and the viral load of HIV in the blood: primary incubation, which lasts 1-2 weeks; acute HIV infection with virus dissemination into lymphoid organs [11]; clinical latency from several weeks up to 20 years; AIDS at which stage CD4⁺ T lymphocyte counts drop under 200 cells/mm³.

Classification of HIV

There are two known HIV species: HIV-1 and HIV-2. HIV-1 was first discovered in 1981 and used to be named as LAV or HTLV-III [12]. Compared to HIV-2, HIV-1 is more virulent, more infectious and constitutes the majority of HIV infections globally. HIV-2 is largely found in West Africa, but it has the relatively lower capacity for transmission and pathogenicity [13].

HIV-1 genome and structure

HIV-1 has a diameter of about 120nm [14]. Each virion contains two identical copies of single positive-stranded RNA genome, which is enclosed by nucleocapsid p6 and p7, matrix, reverse transcriptase and integrase, and envelope. Nucleocapsids p7 and p6 associate with the genomic RNA and protect the RNA from digestion by nucleases. The capsid is surrounded with a matrix with viral protein p17, ensuring the integrity of the virion particle. The envelope is formed when the capsid buds from the host cell, taking some of the host-cell membrane [15, 16]. The envelope glycoproteins gp120 and gp41 form spikes on the virions and play important role in virus-cell attachment [17]. The RNA genome contains seven structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS) and nine genes (gag, pol, and env, tat, rev, nef, vif, vpr, vpu, and tev) [18, 19]. The Tat protein is a transcriptional transactivator for 5' long terminal repeats (LTR) promoter and functions by binding the trans-activation response element (TAR) RNA element. The Rev protein functions to shuttle RNAs from the nucleus and the cytoplasm by binding to the Rev responsive element (RRE). The Nef protein (p27) down-regulates CD4, as well as the MHC class I and class II molecules. The Vpu protein (p16) regulates release of new virus particles from infected cells. LTR promoter has three regions: U3, R, and U5, which contains all the signals for gene expression: enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. The integrated provirus has two LTRs, and the 5' LTR normally acts as an RNA pol II promoter, while 3' LTR works in transcriptional termination and polyadenylation.

HIV-1 life cycle

HIV 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 non-syncytium-inducing (NSI) macrophage-tropic HIV-1 isolates at the early stage of HIV-1 infection, while CXCR4 is used by syncytium-inducing (SI) strain T cell-tropic HIV-1 isolates at later stages of HIV-1 infection [20-22]. 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 system 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 [23, 24]. Viral RNA are transcribed from the integrated proviral DNA and exported into cytoplasm for viral protein synthesis [25, 26]. 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 [27].

Anti-HIV therapy

The anti-HIV therapeutic strategy has been mainly targeted at RT and PR. Some advances have been recently made to target the viral entry and IN. 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; 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 of America has approved a total of 21 anti-HIV-1 drugs, a majority of these drugs are HIV-1 RT and PR inhibitors [28]. Enfuvirtide (Fuzeon) are the two approved HIV fusion inhibitors. Maraviroc binds to CCR5, preventing an interaction with gp120 [29], while Fuzeon binds to gp41 and interferes with its ability to approximate the two membranes [30].

The current treatment for HIV infection is called highly active antiretroviral therapy (HAART) and is a combination of these inhibitors above. HAART is very effective in suppressing HIV-1 replication, but it cannot eradicate the virus from the infected subjects [31]. This therapy cannot achieve the consistent optimal results, because of the non-adherence and non-persistence issues. The reasons for non-adherence and non-persistence could be both psychosocial issues and the complexity of these HAART regimens, such as poor access to medical care, inadequate social supports, and drug abuse; and dosing frequency, meal restrictions or other issues along with side effects. These limitations pose great challenges for long-term HIV/AIDS management [32]. Meanwhile, there have been no vaccines available for HIV/AIDS. Therefore, continued efforts have

been putting into development of new and alternative therapeutic strategies with lower cost, fewer side-effects, and best regimens to manage drug resistance.

Traditional Chinese medicine and its anti-HIV activity

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 [33]. Unlike one single compound-based Western medicine, TCM is a mix of multiple ingredients that are carefully balanced and standardized to maximize its therapeutic efficacy while minimize its toxicity and side-effects. 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 [34-39]. Our studies added *Euphorbiaceae*, *Trigonostema xyphophylloides* (TXE) and *Dipterocarpaceae*, *Vatica astrotricha* (VAD) onto this soon-to-be-rapidly-expanding list. Studies are under way to further fractionate these extracts for identification of the active anti-HIV-1 entry constituents in these extracts and for better characterization of their effects on the interaction between HIV-1 gp120 and CD4/homine receptors CCR5 and CXCR4 [40].

Anti-HIV-1 activity of flavonoids (FL) compounds

FL are also known as Vitamin P or citri, which belongs to the class of plant secondary metabolites. FL are not only the major components in our daily nutrients, but also widely used in medications acting as antioxidants, enzyme inhibitors, and precursors of toxic substances. These compounds possess anti-inflammatory, antithrombotic, anticarcinogenic, and antiviral including anti-HIV-1 activities [41]. The anti-HIV-1 activity of FL compounds been implicated in the inhibition of RT enzyme [42]. Baicalin (BA, 7-glucuronic acid,5,6-dihydroxyflavone) is purified from FL compound that also have known to possess anti-inflammatory and anti-HIV-1 activities through strengthening host defense. Instead of inhibiting the RT enzymatic activity, 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) [43].

The goals of the current study

In a previous study, we show that extracts from two Chinese medicinal herbal plants in Hainan Island, China *Euphorbiaceae*, *Trigonostema xyphophylloides* (TXE) and *Dipterocarpaceae*, *Vatica astrotricha* (VAD) both block HIV-1 replication at the entry [40]. 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 pilosus* (AAP), *Annonaceae* *Oncodostigma hainanense* (AOH) and *Annonaceae* *Dasymaschalon 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, repair fractures, and ameliorate bruises [44]. Several chemical compounds have been isolated and identified from the plant extracts of the *Annonaceae* family and include flavonoids (FL), alkaloids (AL) and acetogenins (AT) [45].

METHODS AND MATERIALS

Preparation of plant extracts

All plants used in this study were collected at the tropical national forest park Bawangling, Hainan Island, P. R. China (Table 1). Scientific names and classification of these plants were validated by Prof. Chen of Hainan Normal University, Haikou, P. R. China. Samples of these plants were kept at the Hainan Provincial Key Laboratory of Tropical Pharmaceutical Herbal Chemistry, Haikou, P. R. China. Plant samples were air dried, grinded and continued to be dried in a pressurized oven at 40 °C and 0.08 MPa. The dried and grinded 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 kept as stock at -20 °C.

Cells, HIV-1 HXB2/YU-2 viruses and chemicals

Jurkat cells 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). G418 (Lonza)(1.25 mg/ml) was included in medium 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 (Lonza) supplemented with 10% fetal bovine serum and 100 units/ml penicillin.

Preparation and infection of HIV-1 pseudotyped viruses

293T cells were plated in a 10 cm dish at a density of 2×10^6 cells per plate and transfected with 20 μ g HIV-Luc plasmid and 4 μ g pHXB2-env, pYu2-env, pVSV-G, or pcDNA3 [40] by the calcium phosphate precipitation method. After 48 hr incubation, the cell culture supernatant was collected, cleared of cell debris, and used for infection. The virus titers were determined by reverse transcriptase assay [46] 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. After 2 hr infection, the culture medium was replaced with the fresh DMEM, continued to incubate for 48 hr and harvested for the Luc activity assay [47, 48].

Reverse transcriptase (RT) assay

The RT activity assay was used to quantitate the relative virus titers and was performed as follows. Briefly, cell culture supernatants were collected, cleared of cell debris by a brief centrifugation, followed by centrifugation at 10,000 g at 4 $^{\circ}$ C for 1 hr to obtain the virion pellet. The virion pellet was suspended in 10 μ l dissociation buffer [30 mM Tris HCl, pH 8.1, 0.1M KCl, 6 mM dithiothreitol (DTT) and 0.6% Triton X-100], followed by three rounds of freezing and thawing. Then, 40 μ l reaction mixture containing 34 μ l

reaction buffer (50 mM Tris HCl, pH 8.0, 2 mM DTT, 100 mM KCl, 10 mM MgCl₂, 0.01% Triton X-100), 5 µl template primer poly(A):oligo(dT) (Roche, Indianapolis, IN) and 1 µl ³H-thymidine (PerkinElmer, Boston, MA) was added to each sample. After one hour incubation under 37 °C, each sample was spotted onto Whatman DE81 filter disc and let air dried at room temperature. This was followed by three times of washes with 2x SSC (0.3 M NaCl and 0.03M sodium citrate) and two times of washes with 100% ethanol. The radioactivity on the filter disc was measured in a liquid scintillation counter and used to express as the relative RT activity [49].

HIV replication assay

One million Jurkat cells 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 cells 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 cells were included as controls.

Flow cytometry analysis

CEM-GFP cells were treated with 10 $\mu\text{g/ml}$ each of the extracts for 3 days or 7 days, and then harvested to determine the GFP expression by flow cytometry.

Data analysis

Results are expressed as mean \pm SEM. 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

The purpose of these experiments was to determine whether any of nine selected traditional Chinese medicinal herbal extracts from *Annonaceae* family have anti-HIV activity and at which steps of the HIV viral cycle they inhibit HIV-1 replication including entry, reverse transcription, gene expression, and post-entry. Different dosages were also evaluated for the anti-HIV effects of the extracts.

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 cells 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 cells and HIV-infected cells without any treatments were also included as controls. The results showed that compared to DMSO control sample, 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) (Fig. 1). However, in the rest of five extracts, we did not detect the significant block in HIV-1 replication (Table 2).

Cytotoxicity of the extracts

Throughout the infection experiments (Fig. 1), we also monitored cell survival of cells of all treatments by trypan blue dye staining. The results showed that at the concentration of 10 $\mu\text{g/ml}$ the cell viability in all extract treatments was similar to that in mock or DMSO-treated cells (Fig. 2). Nevertheless, at a concentration of 100 $\mu\text{g/ml}$, the cell viability in all DMSO-treated and extracts-treated cells began to decrease at day 5 (data not shown). Based on these results, the extract concentration of 10 $\mu\text{g/ml}$ was chosen for all following mechanistic studies.

Dose-dependent inhibition of HIV-1 replication by these extracts

We further determined whether the inhibitory effects on HIV-1 replication of these extracts were dose-dependent. We infected Jurkat cells with HIV-1 viruses and then treated the cells with each of the extracts at a concentration of 5 $\mu\text{g/ml}$ or 1 $\mu\text{g/ml}$. Fresh extracts were added every other day, while the supernatants were collected for the RT activity assay. At the concentration of 1 $\mu\text{g/ml}$, compared to controls, none of the extracts showed significant inhibition of HIV-1 replication, while at the concentration of 5 $\mu\text{g/ml}$, these extracts showed to a different extent inhibitory or delayed effects on HIV-1 replication (Fig. 3). At both 1 and 5 $\mu\text{g/ml}$ concentrations, extracts showed little effects on cell viability (data not shown). These results suggest a dose-dependent inhibition of HIV-1 replication by these extracts and also support our choice of the 10 $\mu\text{g/ml}$ concentration of these extracts throughout our studies.

Block of HIV-1 entry by these extracts

HIV-1 infection begins with HIV-1 envelope gp120 binding to CD4 and chemokine coreceptors CCR5 for M-tropic HIV-1 strains or CXCR4 for T-tropic HIV-1 strains. We then determined whether these extracts had any inhibitory effects at the viral entry step. We took advantage of the replication-defective single round HIV-Luc reporter system [50]. In this reporter system, HIV-1 *env* gene is nonfunctional and the firefly luciferase gene is inserted into and inactivates 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 then pre-treated U87.CD4.CXCR4 or U87.CD4.CCR5 cells for 1 hour with 10 µg/ml each of extracts before we infected these cells with the pseudotyped viruses (HXB2 for U87.CD4.CXCR4; YU-2 for U87.CD4.CXCR5). We also included pseudotyped viruses without any envelope and vesicular stomatitis virus envelope glycoprotein (VSV-G)-pseudotyped viruses as negative and positive controls, respectively. Compared to the DMSO treatment, all extract treatments showed little luciferase activity in HXB2-infected U87.CD4.CXCR4 cells (Fig. 4A) or YU-2-infected U87.CD4.CCR5 cells (Fig. 4B), suggesting that pre-treatment of these extracts completely blocked entry and infection of both T- and M-tropic HIV viruses. 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).

No effects of the extracts on HIV-1 infectivity

To further confirm that these extracts inhibited HIV-1 replication through masking the receptors on the target cells during the entry step, we determined if these extracts have effects on HIV-1 infectivity. To this end, we incubated HXB2- or YU-2-pseudotyped HIV-Luc viruses with the extracts 37 °C for 2 hr. We then recovered the viruses by centrifugation. We then infected U87.CD4.CXCR4 or U87.CD4.CCR5 cells with these viruses. Compared to DMSO treatment, these extract treatments showed little differences of the luciferase activity (Fig. 5), further indicating that these extracts have no effects on HIV-1 itself.

No effects of the extracts on HIV-1 post-entry

To determine whether these extracts had inhibitory effects on any post-entry events, we first 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 DMSO treatment, none of the extract-treated cells showed significant decreases in the luciferase activity (Fig. 6), suggesting that these extracts have no effects on any post-entry events and further confirming that these extracts inhibit HIV-1 replication at the entry step of the viral 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 cDNA molecule. Thus, we next determined whether these extracts have any direct inhibitory effects in 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 (Fig. 7).

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 first determined whether these extracts affect the HIV-1 LTR promoter activity. 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 [51]. We treated these cells with 10 $\mu\text{g/ml}$ 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 (Fig. 8), suggesting that the extracts from AAP-leaves, AAP-stems, AAP-roots and ADR-roots have no inhibitory effects on HIV-1 LTR promoter activity and gene transcription.

DISCUSSIONS

In this study, we modified the HIV-1 replication system, a well-established system published in our previous work to screen nine new TCM herbal extracts from Hainan Island, China to determine 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. These species are well-known working in antifungal, bacteriostatic, antimalarial, and cytostatic capability of some chemical constituents of the leaves and stems. The chemical compounds, including flavonoids(FL), alkaloids(AL) and acetogenins(AT), have been extracted [45]. Baicalin (BA) is purified from FL compound that have known to possess anti-inflammatory and anti-HIV-1 activities. BA inhibits HIV-1 infection by interfering with viral entry, through the interaction between HIV-1 envelope proteins and the cellular CD4 and chemokine receptors (both CD4/CXCR4 and CD4/CCR5) [43, 52, 53].

We found that AAP-leaves, AAP-stems, AAP-roots and ADR-roots have completely blocked HIV-1 replication at concentration of 10 g/ml (Fig. 1 and Fig. 2). Instead, we did not see any significant HIV-1 replication inhibitions in the rest of other samples (data shown as percentages in Table 2). Dosage experiments were included to detect whether the different would affect anti-HIV. The data indicated that the extracts have no inhibitions on HIV-1 replication at concentration of 1 µg/ml, while at concentration of 5 µg/ml, AAP—leaves and AAP-roots treated cells showed significant inhibition compared

to DMSO-treated cells on HIV-1 replication (Fig. 3). Following this results, we detected the mechanisms of these extracts by testing direct effects on LTR promoter activities, RT enzymatic activities and HIV-1 virus entry. However, we did not detect any effects of these extracts on LTR promoter activities (Fig. 8). On other hand, we showed the significant inhibition of HIV-1 RT enzymatic activities by treating RT with AAP-leaves and AAP-roots directly (Fig. 7). Moreover, we also determined that all four sample potently blocked HIV-1 from entering its target cells (Fig. 4). Shortly, these results indicated that four samples have anti-HIV infection: AAP-stems and ADR-roots block HIV-1 replication through interfering with HIV-1 entry, while AAP-leaves and AAP-roots block HIV-1 replication by both interfering with HIV-1 RT enzymatic activities and their entry. To further boost this conclusion, we performed the experiments of HIV-1 infectivity and post-entry. However, there were no effects of these extracts on both HIV-1 infectivity and post-entry (Fig. 5 and Fig. 6).

Theoretically, corresponding to the HIV-1 virion cycle, the extracts should have inhibitions to HIV-1 post-entry when they block RT enzymatic activity. However, from the results, AAP-leaves and AAP-roots had statistically inhibitions on RT enzymatic activity, while neither of them had effects on HIV-1 post-entry (Fig. 6 and Fig. 7). This could be because the extracts were not able to enter the U87.CD4.CCR5/CXCR4 cells, but interfere with the virion entry. This could also be one possible explanation on LTR promoter experiment that the extracts were not capable entering CEM-GFP cells. (Fig. 8) Taken all together, AAP-leaves, AAP-stems, AAP-roots and ADR-roots have completely blocked HIV-1 replication at concentration of 10 µg/ml, while AAP-leaves and AAP-

roots delayed HIV-1 replication at concentration of 5 µg/ml. These four extracts achieved this conclusion by interfering with HIV-1 entry step. Further studies are required to indicate whether they inhibited both T cell tropic (X4) and monocyte tropic (R5) HIV-1 Env protein mediated fusion with cells expressing CD4/CXCR4 or CD4/CCR5, or inhibit binding of HIV-1 gp120 to CD4, even though flavonoid baicalin interfere with the interaction of HIV-1 Env and chemokines. Furthermore, AAP-leaves and AAP-roots can also inhibit HIV-1 RT enzymatic activities. These results are also consistent with the previous data that chemical compound FL inhibits HIV-1 infection at the level of viral entry and RT enzymatic activity [43].

The goal of anti-HIV medication treatment is to reduce the amount of virus in a person's body and prevent destruction of the immune system. They help HIV/AIDS infected patients live a longer life. There are twenty-one anti-HIV medications currently in market approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV. HIV patients are required to use these medications in combination. However, all the antiretroviral drugs have different levels negative side effects, from mild to life-threatening [32]. In contrast, traditional Chinese medicinal herbal extracts have a much short development cycle, much fewer side-effects and relatively inexpensive due to their natural origin.

With the history of 2000-3000 years, TCM cure several illnesses by their unique system. The basic TMC treatment principle with HIV/AIDS is to support the body's energetic basis by nourishing the deficiencies while eliminating the deeper underlying pathogens

such as Toxic Heat, Phlegm, and Blood Stasis [8]. There are a few of Chinese herbs using for treating HIV/AIDS patients: Immunity-boosting herbs, such as astragalus, echinacea, and ginkgo are also used to help revive an ailing immune system, and certain herbs such as garlic are used to battle bacteria and viruses in HIV/AIDS treatments. Deglycyrrhizinated licorice can soothe the mouth and throat ulcers that often accompany full-blown AIDS. In this study, I detected another four possible samples from *Annonaceae* Family: AAP-leaves, AAP-stems, AAP-roots and ADR-roots, to inhibit HIV-1 replication through interrupting HIV-1 replication entry. However, to develop new drugs and improved formulations from Chinese herbs, many challenges applied due to their lack of active ingredient [54].

TABLES

NO.	Plant (family, species)	Sampling location	Sampling parts	Medicinal usage
1	<i>Annonaceae, Artabotrys pilosus</i> Merr. et Chun	Bawangling	Leaves	Anti-Malaria, TB
2	<i>Annonaceae, Artabotrys pilosus</i> Merr. et Chun	Bawangling	Stems	Anti-Malaria, TB
3	<i>Annonaceae, Artabotrys pilosus</i> Merr. et Chun	Bawangling	Roots	Anti-Malaria, TB
4	<i>Annonaceae, Oncodostigma hainanense</i> (Merr.) Tsiang et P.T.Li	Bawangling	Fruit	Bleeding, fractures, bruises
5	<i>Annonaceae, Oncodostigma hainanense</i> (Merr.) Tsiang et P.T.Li	Bawangling	Leaves	Bleeding, fractures, bruises
6	<i>Annonaceae, Oncodostigma hainanense</i> (Merr.) Tsiang et P.T.Li	Bawangling	Stems	Bleeding, fractures, bruises
7	<i>Annonaceae, Dasymaschalon rostratum</i> Merr. et Chun	Bawangling	Roots	Anti-tumor
8	<i>Annonaceae, Dasymaschalon rostratum</i> Merr. et Chun	Bawangling	Stems	Anti-tumor
9	<i>Annonaceae, Dasymaschalon rostratum</i> Merr. et Chun	Bawangling	Leaves	Anti-tumor

Table 1. The information of the nine Chinese herbal medicinal extracts. These extracts are from *Annonaceae* family, found in national tropical forest parks in Hainan island Bawangling, P. R. China. Plant samples were first air dried, grinded and continued to be dried in a pressurized oven at 40 °C and 0.08 MPa. The dried and grinded materials were 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 a desiccator.

samples/dates	Day 4	Day 6	Day 8	Day 10	Inhibit HIV replication?
DMSO	3±0.3	12±2.7	56±8.8	100	
AAP-leaves	0.62±0.1	2.2±1.2	2.0±0.7	2.5±1.5	Yes
AAP-stems	0.68±0.3	0.8±0.2	2.9±0.8	2.8±0.1	Yes
AAP-roots	0.52±0.31	0.48±0.01	2.5±0.9	1.4±0.2	Yes
AOH-fruit	5.12±1.1	86.8±8.3	121±23	178±12	No
AOH-stems	5.0±0.06	67±6	102±32	94±8	No
AOH-leaves	0.4±0.01	19±3.2	77±33	75±21	No
ADR-leaves	2.1±0.3	63±0.5	115±7.4	107±10	No
ADR-stems	2.4±0.3	71±4.6	79±18	122±8	No
ADR-roots	0.17±0.04	0.6±0.5	2.6±1.3	2.3±0.7	Yes

Table 2. The average percentage of HIV-1 replication with nine extracts treatments based on three independent experiments. The RT assay readings of each sample were collected every other day. The percentage was considered as 100% when HIV-1 replication of DMSO sample reached to the peak. Instead, the rest of other samples with extracts treatments were calculated and converted into percentages based on DMSO. The data were mean \pm SEM of three independent experiments.

FIGURES

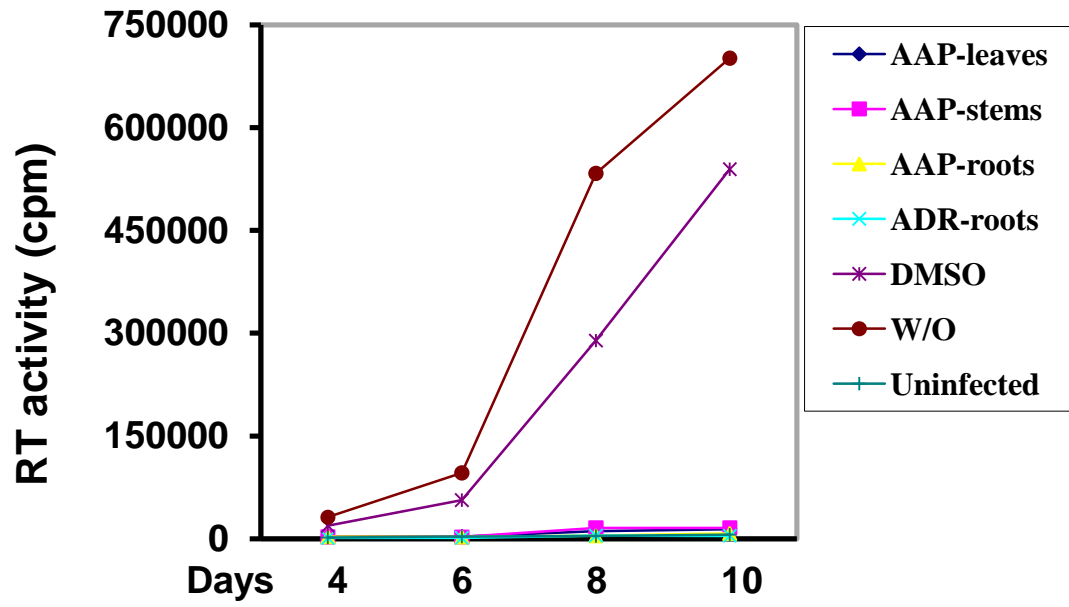


Figure 1. Effects of extracts on HIV-1 replication. One million Jurkat cells were infected with HIV-1 HXB2 corresponding to a 10,000 cpm RT activity for 24 hr and then treated with 10 µg/ml fresh extracts from the leaves, stems and roots of Annonaceae, *Artabotrys pilosus* (ARP-leaves, stems, and roots) 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 and HIV-infected cells without any treatments were also included as controls. These data were representative of three independent experiments.

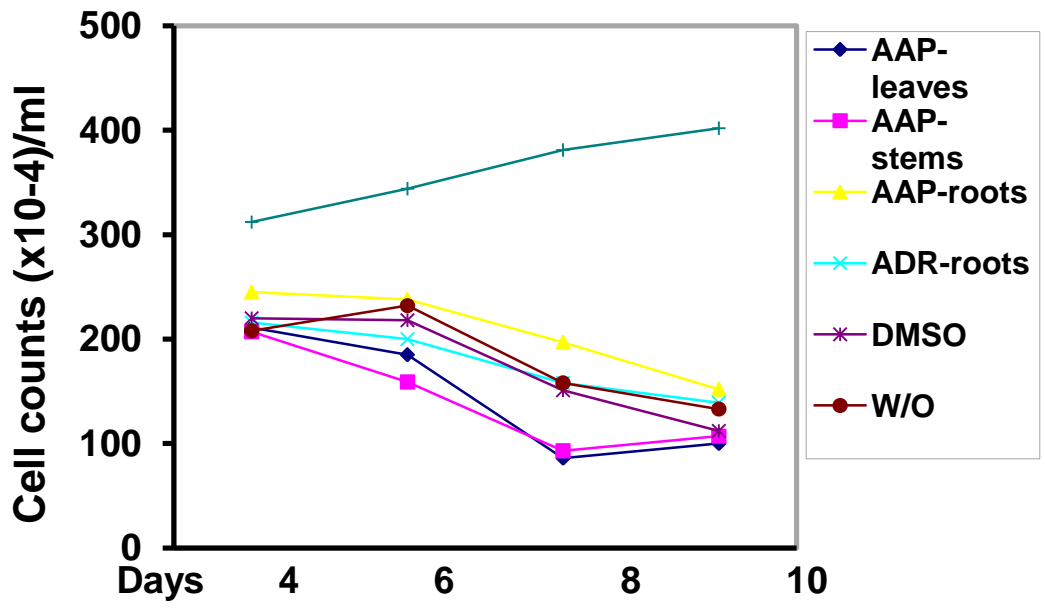
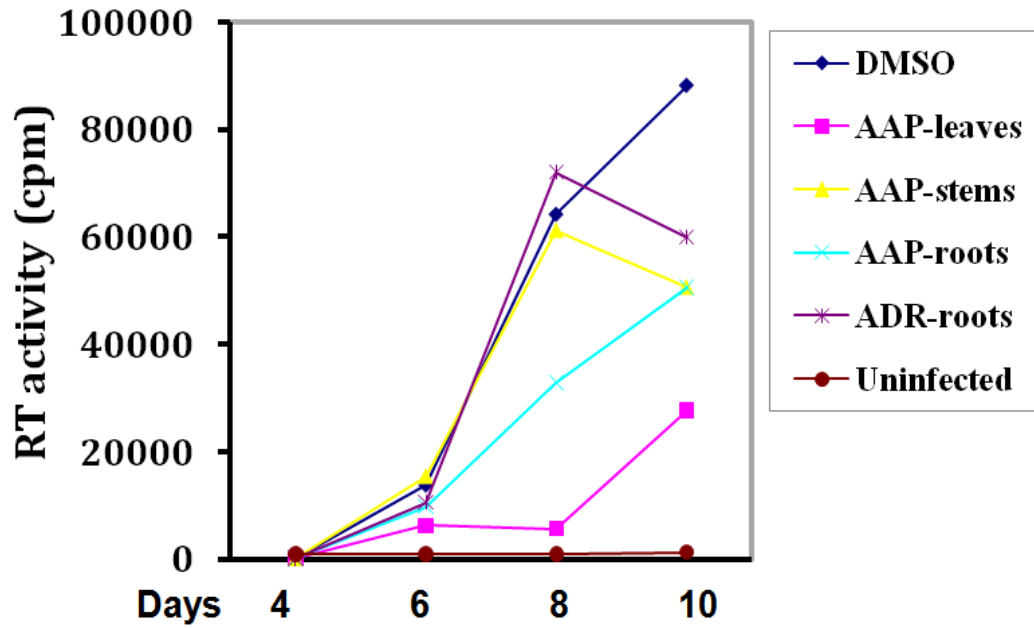


Figure 2. Effects of extracts on Jurkat cell survival. Jurkat cells were infected and treated as in Fig. 1. At each time point, aliquot of cells was collected, stained with trypan blue dye, and then counted for the cell viability. These data were representative of three independent experiments.

A.



B.

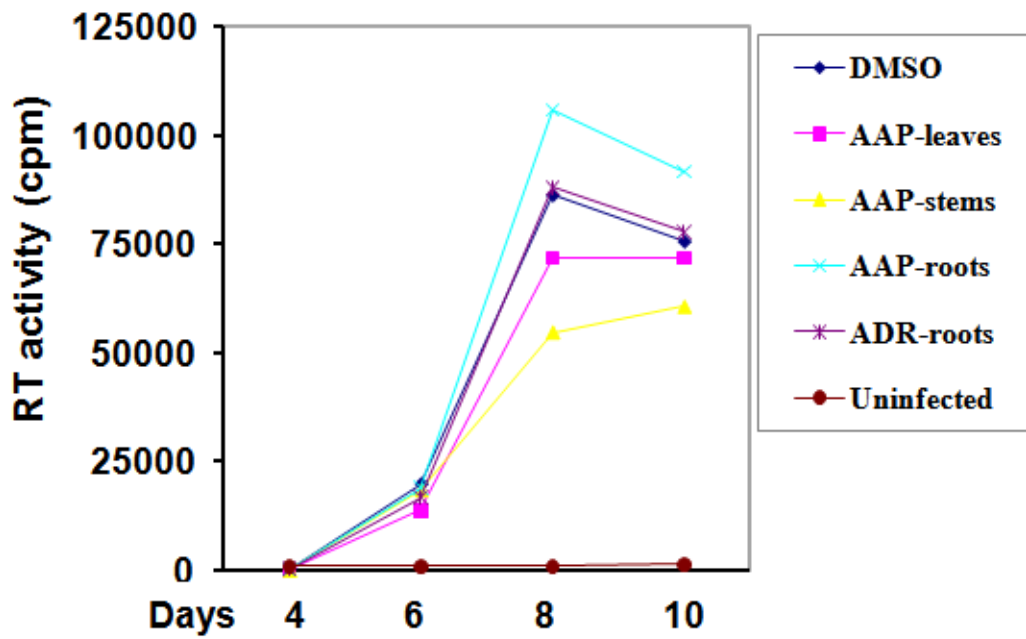
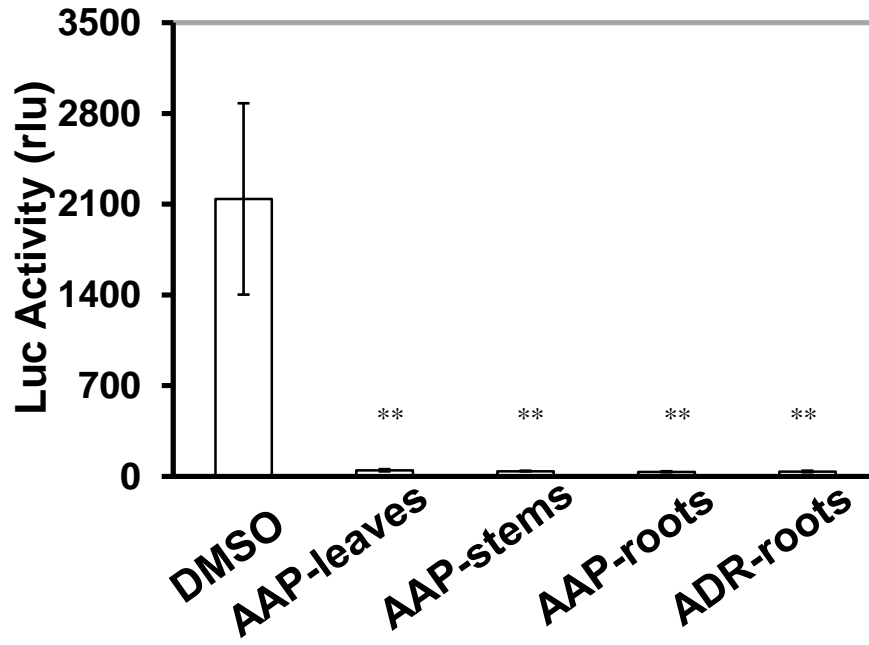


Figure 3. Effects of extracts on HIV-1 replication at different doses. Jurkat cells were infected as in Fig. 1. Then the cells were treated with 5 $\mu\text{g/ml}$ (A) or 1 $\mu\text{g/ml}$ (B) fresh extracts from AAP-leaves, stems, and roots and ADR-roots every other day. RT assay was applied as in Fig.1. DMSO was used as a vehicle control and mock-infected cells was included as a negative control. These data were representative of three independent experiments.

A.



B.

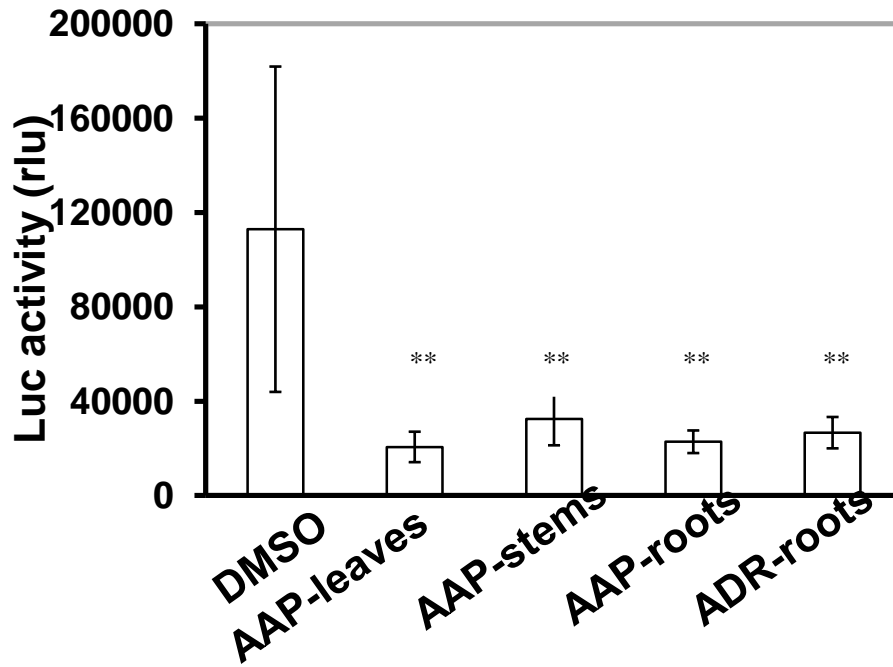
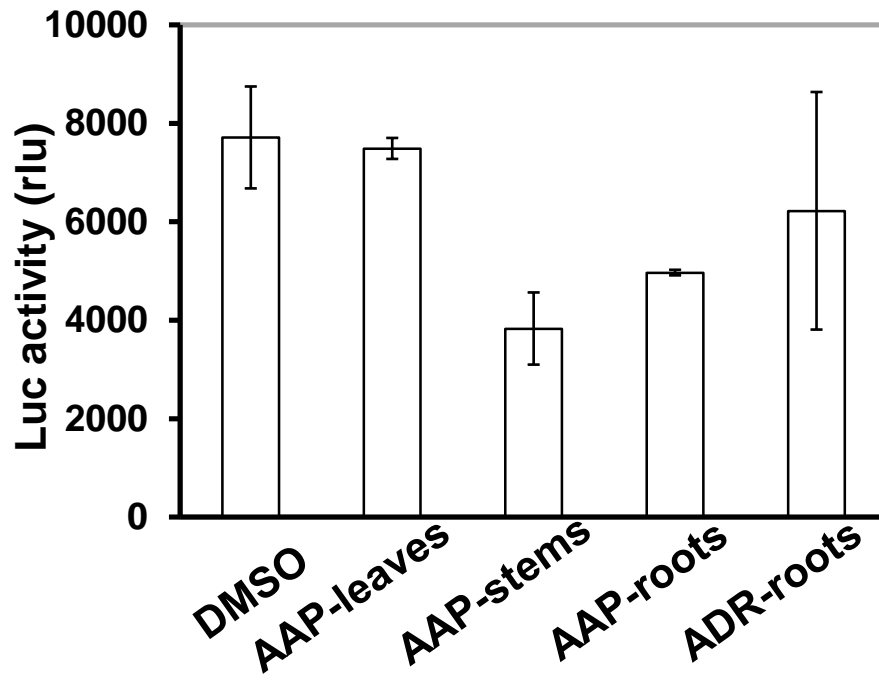


Figure 4. Effects of extracts on HIV-1 entry. A. U87.CD4.CXCR4 cells were treated with 10 $\mu\text{g/ml}$ each of the extracts for 1 hr and then infected with HIV-Luc viruses pseudotyped T-tropic HIV-1 HXB2 envelope. B. Similar experiments were performed with U87.CD4.CCR5 cells and HIV-Luc viruses pseudotyped M-tropic HIV-1 YU-2 envelope. HIV-Luc viruses pseudotyped with VSV-G envelope and no envelope were included as the positive and negative infection controls, respectively (data not shown). The data were mean \pm SEM of triplicate experiments.

A.



B.

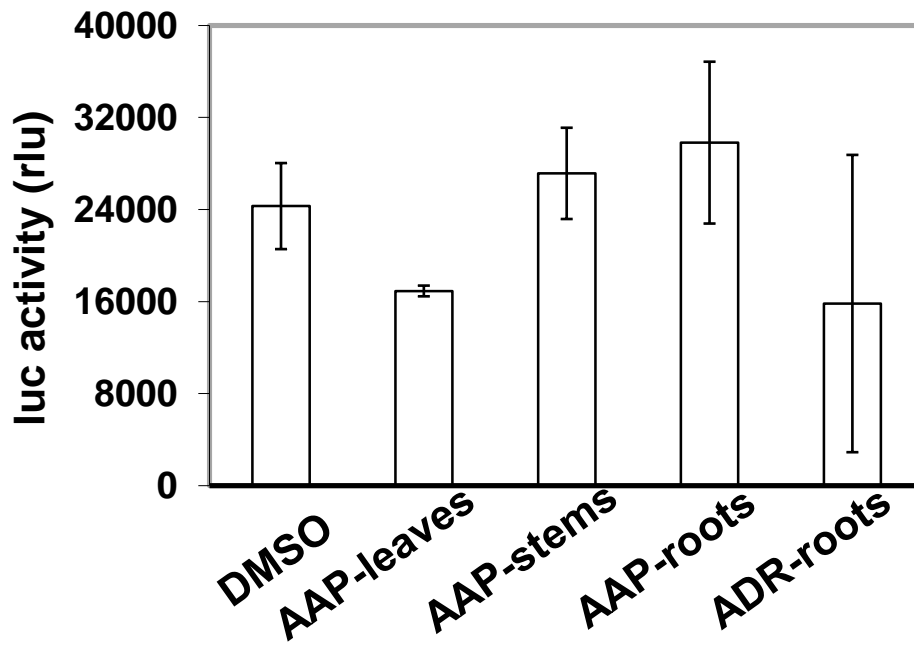
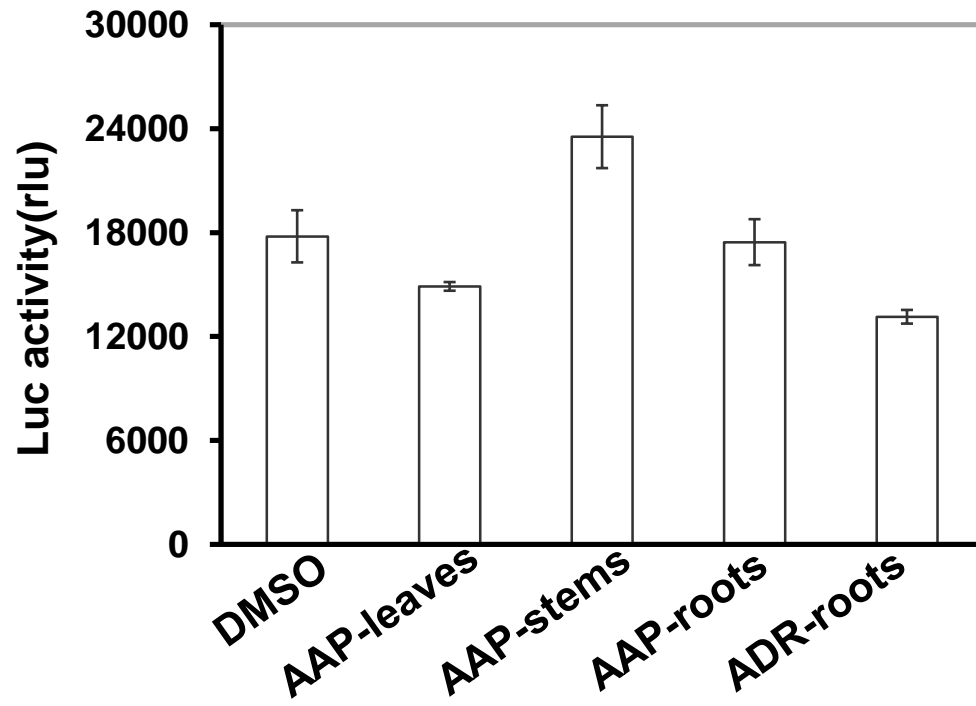


Figure 5. Effects of extracts on HIV-1 infectivity. HIV-Luc virus pseudotyped with HXB2 (A) or YU-2 envelope (B) was first incubated with 10 $\mu\text{g/ml}$ each of the extracts at 37 $^{\circ}\text{C}$ for 2 hr. The virus was recovered by centrifugation and then used to infect either U87.CD4.CXCR4 (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 \pm SEM of triplicate experiments.

A.



B.

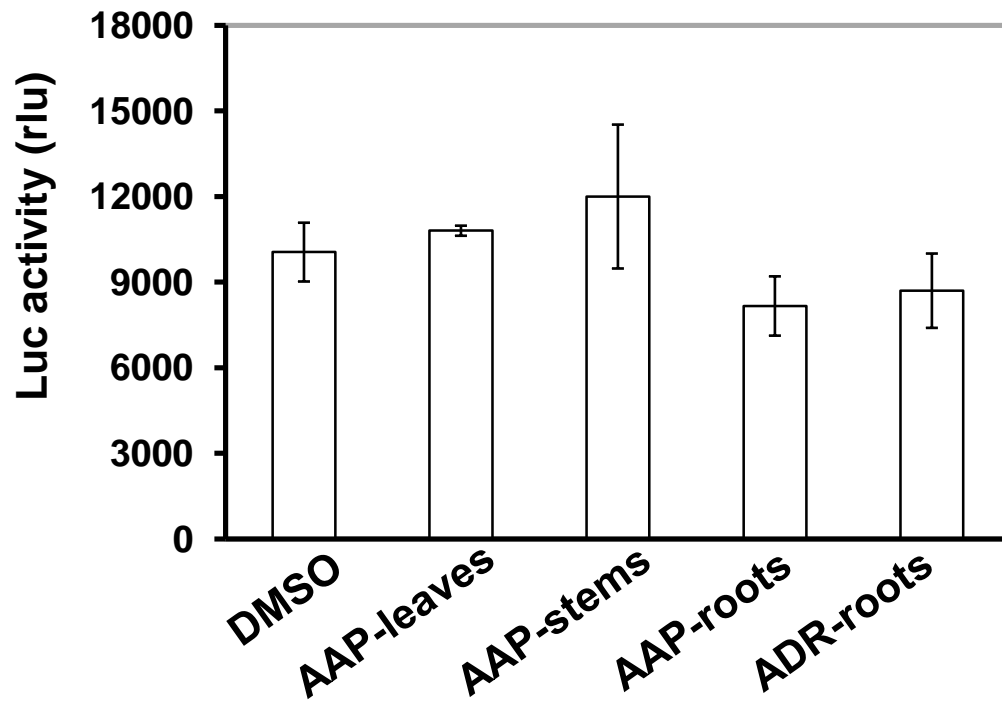


Figure 6. Effects of extracts on HIV-1 post-entry. U87.CD4.CXCR4 and U87.CD4.CCR5 cells were first infected with HIV-Luc virus pseudotyped with HXB (A) or YU-2 envelope (B). Following medium change, the cells were treated with 10 $\mu\text{g/ml}$ each of the extracts for 48 hr and then harvested for the luciferase reporter gene assay. The data were mean \pm SEM of triplicate experiments.

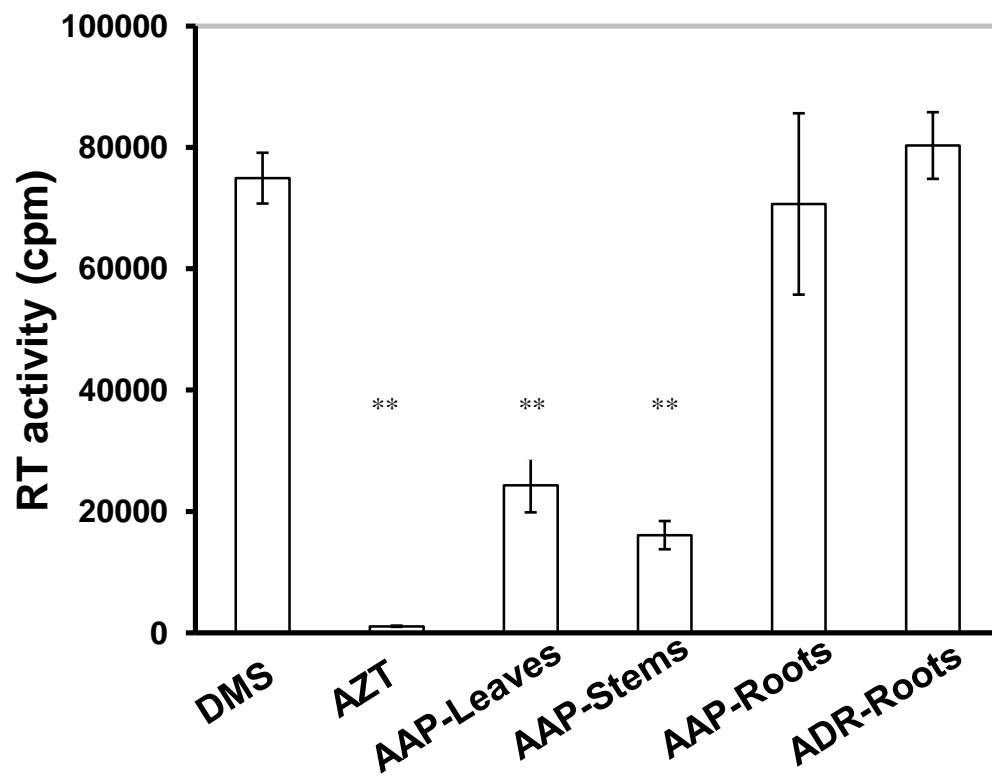
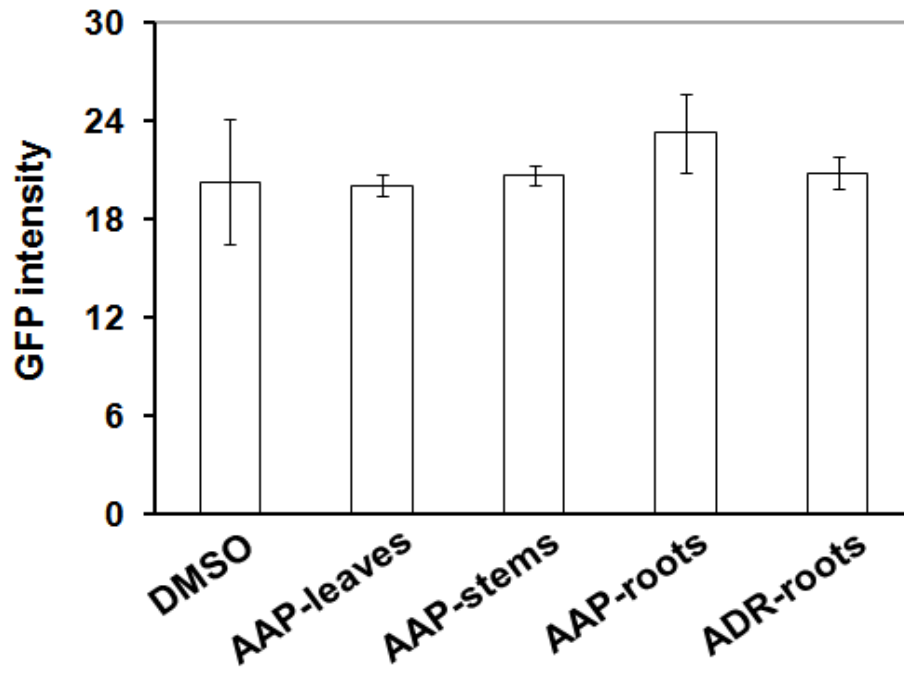


Figure 7. Effects of extracts on the HIV-1 RT activity. Purified HIV-1 virions were incubated with 10 µg/ml each of the extracts for the RT activity assay. The RT inhibitor AZT (5 µM) was included as a positive control, while DMSO was used as the vehicle control for the extracts.

A.



B.

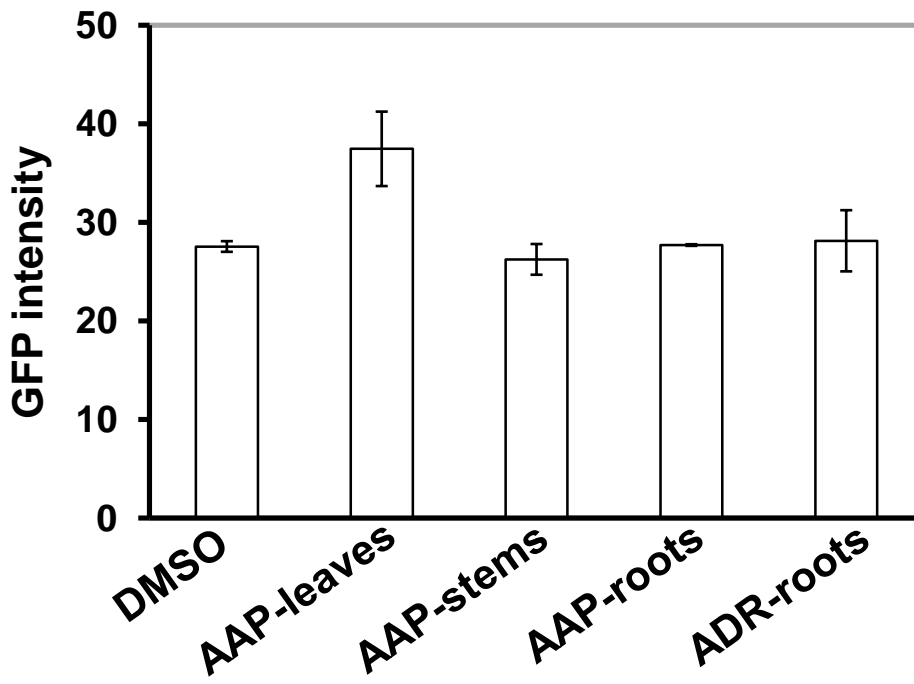


Figure 8. Effects of extracts on the HIV-1 long terminal repeat (LTR) promoter activity. CD4⁺ T lymphocytes CEM stably expressing green fluorescence protein (GFP) under the control of HIV-1 LTR promoter were treated with 10 µg/ml each of the extracts for 3 days (A) and 7 days (B). Then, the cells were 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.

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CURRICULUM VITAE

Ting Wang

Undergraduate Degree

Department of Microbiology and Molecular biology
University of Sheffield
Sheffield, U.K.
September 2003 – June 2006

Graduate Degree

Master of Science
Department of Microbiology and Immunology
Indiana University
Indianapolis, U.S.
September 2008 – December 2010