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Salviae and Cinnamomi Herbal Medicines have Antiviral Activity against a Broad Range of Human Immunodeficiency Virus

Fu-Shi Quan[1], Young Jae Lee[2], Richard W Compans[3] and Sang-Moo Kang[4]*

[1]*Department of Medical Zoology, Kyung Hee University School of Medicine, Seoul, Korea*

[2]*College of Veterinary Medicine, Jeju National University, 66 Jejudaehakno, Jeju 690-756, Republic of Korea*

[3]*Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA*

[4]*Center for Inflammation, Immunity & Infection, Institute of Biomedical Sciences, Georgia State University, Atlanta, GA 30303, USA*

***Corresponding author:** *Sang-Moo Kang, Center for Inflammation, Immunity & Infection, Institute of Biomedical Sciences, Georgia State University, Atlanta, GA 30303, USA, Tel: 404-413-3588, E-mail: skang24@gsu.edu*

Abstract

Development of broadly effective anti-human immunodeficiency virus (HIV)-1 compounds is a high priority since the number of individuals having HIV infections continues to grow worldwide and vaccines are not available yet. From herbal extracts commonly used in humans, *Salviae miltiorrhiza* (Salviae), *Cinnamomi Ramulus* (Cinnamomi), *Astragali Radix* (Astragali), *Asari Radix et Rhizome* (Asari), *Panax Ginseng* (Korean red ginseng), we screened candidate herbal medicines and investigated their antiviral activity against a broad range of HIV-1 strains including clades A and C primary isolates that are dominantly found in Africa and India. Antiviral activity was determined based on the levels of inhibiting viral replication of infectious HIV-1 or infectivity of pseudovirion particles. Ginseng but not Astragali and Asari showed low to moderate levels of antiviral activity against HIV-1 strains. Most significantly, we found that Salviae and Cinnamomi showed a potent antiviral activity against antigenically and geographically different strains of HIV-1. In particular, Salviae extract reduced over 90% of infectivity of all strains tested including clades A, B, and C. The results in the present study provide evidence that Salviae and Cinnamomi extracts may have the potential to be developed for the discovery of novel anti-IV-1 microbicides or drugs which are applicable to a broad range of HIV-1 strains found in the developing and developed countries.

Keywords

HIV-1, Antiviral activity, Herbs, Salviae, Cinnamomi

Introduction

Human immunodeficiency virus type-1 (HIV-1) is the cause of a major pandemic, seriously threatening the public health worldwide particularly in developing countries. Despite enormous efforts, effective vaccines against HIV-1 are not available yet for human use and the number of individuals having HIV infections continues to grow. Therefore, there is an urgent need to develop alternative means to prevent or control HIV infections. Herbal medicines including ginseng are widely used in the United States, with approximately one quarter of adults reporting the use of an herb to treat a medical illness. Ginseng is reported to have a wide range of therapeutic and pharmacological benefits. Ginsenosides, the major pharmacologically active ingredients of ginseng, appear to be responsible for most of the activities of ginseng including vasorelaxation, antioxidation, anti-inflammation and anti-cancer {Lu, 2009 #34}. Natural products have provided a source for the discovery of many novel therapeutic agents. Approximately one third of human drugs were originally derived from plant sources [1]. *Astragali Radix* is used in treatment of abscess, ulcers and skin eruptions. The root extracts of *Salviae miltiorrhiza* are commonly used in China, Japan, the United States, and European countries. The root extracts of *Salviae miltiorrhiza* are known to promote the flow of bloodstream and used for the treatment of cardiovascular and cerebrovascular diseases. In China, *Salvia miltiorrhiza* is used to treat variety of diseases such as angina pectoris, myocardial infarction, hypertension, hyperlipidemia, and acute ischemic stroke. *Cinnamomi Ramulus* is used for gas (flatulence), muscle and stomach spasms, preventing nausea and vomiting, diarrhea, infections, the common cold, and loss of appetite. *Asari Radix et Rhizome* is widely used as an antitussive and analgesic agent in the treatment of cough and dyspnoea, headache, toothache, rheumatoid arthritis and sinusitis. Although traditional Chinese medicines are widely used in humans, our knowledge on their therapeutic effects on human disease is very limited. It is suggested that research efforts should be highly encouraged towards identifying novel herbal products that have efficacy for treating or preventing disease. It was previously demonstrated that HIV-1 infected individuals who had taken Korean red ginseng showed significantly improved clinical parameters including slower decreases in CD4 T cells, lower copy numbers of HIV RNA and a decrease in serum soluble CD8 antigen, resulting in fewer cases requiring anti-retroviral drug treatments and hospital admission [2].

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In an effort to understand the potential mechanism by which ginseng exhibits its positive clinical effects in HIV-1 infected individuals, we tested the possibility of potential anti- HIV-1 activities of ginseng and several other common herbal medicines that are routinely prescribed to healthy individuals and patients.

Material and Methods

Virus and cells

HIV-1 89.6 strain and single-cycle replication-competent HIV-1 pseudovirions containing envelope proteins from diverse HIV-1 primary isolates of clades A, B, and C were used as described in table 1. JC53-BL cells was used which are derivative of HeLa cells expressing β-galactosidase from an HIV-1 LTR promoter were used. Pseudovirions HIV-1 containing env gene YU2 (NIH AIDS reagent program) and consensus HIV-1 env of clade B (ConB) were used.

Herbs and herbal extract preparation

Korean red ginseng root powder (*Panax Ginseng*, The Korean Ginseng Co. Ltd.) that was the same type of ginseng products prescribed for HIV-1 infected patients was used for preparation of extracts. Other herbal medicines used in this study include the roots of Salviae (Botanical Name Salvia miltiorrhiza as described [3-6], the branches of Cinnamomi (*Cinnamomi Ramulus*) [7], the roots of Asari (*Asari radix et rhizome*), and the roots of Astragali (*Astragali Radix*) (Figure 1A, Figure 1B, Figure 1C, Figure 1D, Figure 1E). These herbal medicines are routinely prescribed by licensed oriental medicine doctors. Extracts of herbal drugs were prepared and kindly provided by Dr. In- Seok Jung (Grace Acupuncture and Herbs Clinic, Duluth, GA, USA). Briefly, dried herb plants were grounded into small pieces. Water extracts of herbs were prepared as follows. Herbs were mixed with distilled water and then heated to 80 - 90°C for 2 h. The aqueous extracts were 0.45 μm membrane-filtered and kept at 4°C until use (Ginseng: 2.5 mg/ml, Salviae: 4.2 mg/ml, Cinamomi: 1.3 mg/ml, Astragali: 0.76 mg/ml, Asari: 1.2 mg/ml based on dried herbal plant weight after removing undissolved solid plant materials). Approximately less than 20% of the weight of dried herbs was extracted into the aqueous solution. Batch to batch preparations were maintained consistent. Since an herbal extract contains a mixture of various components including proteins and organic compounds, the protein concentrations were determined and normalized for cytotoxicity assays.

Table 1: Antiviral activities of herbal extracts against diverse HIV-1 primary isolates of clades A, B, and C.

			Anti-HIV-1 titers (50% inhibition dose)[1]			
Viruses	**Clade**	**Gingseng**	**Salviae**	**Cinnamomi**	**Asari**	**Astragali**
SHIV-89.6P	B	22	119	150	< 20	< 20
SF162.LS	B	< 20	129	348	< 20	< 20
6535.3	B	24	489	514	< 20	< 20
QH0692.42	B	125	433	255	< 20	< 20
SC422661.8	B	36	6,768	763	< 20	< 20
PVO.4	B	321	12,828	1,008	< 20	< 20
Du123.6	C	27	945	403	< 20	< 20
Du156.2	C	38	1,079	370	< 20	< 20
Q23.17	A	26	309	290	< 20	< 20
Q168.a2	A	20	431	298	< 20	< 20
SVA-MLV	Control	24	90	95	< 20	< 20
			Anti-HIV-1 titers (90% inhibition dose)[2]			
SHIV-89.6P	B	< 20	68	64	< 20	< 20
SF162.LS	B	< 20	62	50	< 20	< 20
6535.3	B	< 20	85	68	< 20	< 20
QH0692.42	B	< 20	74	39	< 20	< 20
SC422661.8	B	< 20	247	79	< 20	< 20
PVO.4	B	< 20	587	134	< 20	< 20
Du123.6	C	< 20	118	80	< 20	< 20
Du156.2	C	< 20	186	84	< 20	< 20
Q23.17	A	< 20	82	72	< 20	< 20
Q168.a2	A	< 20	71	63	< 20	< 20
SVA-MLV	Control	< 20	26	29	< 20	< 20

[1]Dilutions of herbal extracts at which relative luminescence units were reduced to 50% compared to virus control wells without the test sample.

[2]Values are the sample dilution at which relative luminescence units were reduced 90% compared to virus control wells (not containing any test sample).

Virus neutralization assay

JC53-BL cells that were used for neutralization activity assays of HIV-1 were incubated with various concentrations of herbal extracts to determine the cytotoxicity of herbal extract. After culture for 3 days, we counted the live cells (unstained) based on resistance to penetration of trypan blue dye, indicating the membrane integrity, and the dead cells (stained). Use of pseudovirions to assess neutralizing activities of antibodies against HIV-1 primary isolates *in vitro* has been well described [8-11]. In brief, plasmid pHIVSG3 (a kind gift from Dr. Beatrice Hahn, University of Alabama at Birmingham) containing the entire virus genome with a deleted env gene was mixed with a plasmid containing the HIV env gene YU2 (NIH AIDS reagent program) or consensus HIV-1 env of clade B (ConB), and transfected into 293T cells using calcium phosphate precipitation. Then pseudovirion particles were harvested at day 2 post transfection and used for anti-HIV-1 activity assays. The codon optimized ConB sequence was described [12] and obtained from Dr. Beatrice Hahn.

Toxicity of herbal extract in mice

Regarding safety and toxicity concerns of herbal extracts, mice were injected subcutaneously daily for 10 days (250 μg per mouse based on the amount of protein) and did not show any sign of side effects or changes in body weight. From the cytotoxicity results, anti-HIV-1 activities were performed in a range of 20 to 100 fold lower than maximum concentrations with no observed cytotoxicity.

Results and Discussion

Initially we determined virucidal activities of several herbal extracts including ginseng against an infectious virus, the HIV-1 89.6 strain. The neutralization assay was based on an indicator cell line,

Figure 1: Herbal medicines used in this study. A). Ginseng (*Panax Ginseng*), B). Cinnamomi (*Cinnamomi Ramulus*), C). Salviae (*Salviae miltiorrhiza*), D). Astragali (*Astragali Radix*), E). Asari (*Asari Radix et Rhizome*).

JC53-BL, which is a derivative of HeLa cells expressing β-galactosidase from an HIV-1 LTR promoter [8,9]. Infection with HIV-1 induces the expression of β-galactosidase in infected cells and blue spots were counted as a measure of viral infection. The detailed procedure was previously described [13,14]. Ginseng showed a moderate level of anti-HIV-1 89.6 virucidal activity (Figure 2A). Interestingly, Cinnamomi and Salviae herbal extracts showed much more potent anti-HIV-1 activities. In particular, Salviae exhibited antiviral activity against HIV-1 at as low as 0.02 μg/ml concentration whereas Asari and Astragali did not show any significant levels of antiviral activity. Only minimal cytotoxicity of 5-10% cell death was observed up until the 1000 μg/ml concentrations of ginseng extract. Similarly, no visible cytototoxicity of JC53-BL cells was observed after incubation with Cinnamomi, Salviae, Astragali, Asari herbal extracts up to 500 μg/ml concentrations.

From the unexpected promising results of virucidal effects of Cinnamomi and Salvia, we further extended the study of their antiviral effects on different HIV-1 strains. As shown in Figure 2B and 2C, ginseng showed a moderate level of antiviral activity against HIV-1 YU2 and ConB pseudotypes. Low levels of antiviral activity were observed in Asari herbal extracts but not in Astragali. Surprisingly, highest levels of antiviral activity against both YU2 and ConB strains were observed in Salviae herbal extract. Cinnamomi extract also showed high antiviral activity against both YU2 and ConB strains but slightly lower than Salviae. Elicitation of broadly neutralizing antibodies against HIV-1 is a challenging and difficult hurdle. Thus, an antiviral drug that is broadly reactive against different clades of HIV-1 would be desirable. We tested anti-viral activities of herbal extracts against a more extended panel of primary isolates of HIV-1 including clades A, B and C, which are the strains causing serious AIDS epidemic in developing countries. The herbal extracts were tested against one simian human immunodeficiency virus (SHIV) and nine primary HIV-1 isolates (Table 1). The HIV-1 stocks tested were human PBMC-grown SHIV-89.6P and 293T cell-produced pseudotyped viruses with Env of primary HIV-1 isolates (Table 1).

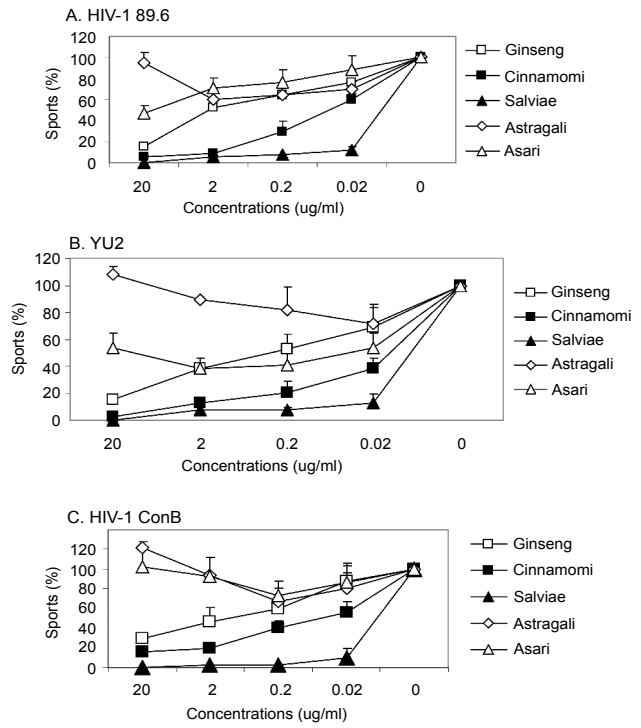

Figure 2: Virucidal activity of herbal extracts against infectious live HIV-1 89.6 and pseudovirions containing HIV-1 envelope protein from primary isolates. Approximately 100 infectious particles of HIV-1 89.6, pseudovirions of HIV-1 YU2 or HIV-1 ConB were incubated with various concentrations of herbal extracts and transferred to monolayer cell cultures of JC53 cells. The remaining infectious centers were counted under the microscope and results are represented as percentile compared to the control well without herbal extracts. (A) HIV-1 89.6, (B) Pseudovirion HIV-1 YU2, (C) Pseudovirion HIV-1 ConB.

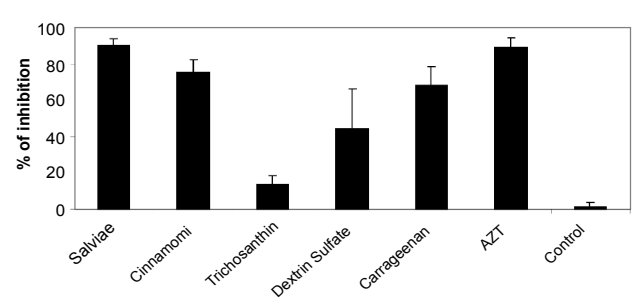

Figure 3: Comparison of virucidal activities for HIV-1 primary isolate SF162 pseudovirions. Levels of inhibition were presented compared to that of a control well with medium alone. Concentrations of test samples are as follows: Salviae (0.2 μg/ml), Cinnamomi (0.2 μg/ml), Trichosanthin (0.2 μg/ml), Dextran Sulfate (1 μg/ml), Carrageenan (κ and λ) (0.5 μg/ml), AZT (3'-azido-3'-deoxythymidine; Zidovudine, 0.032 μmole /ml, an anti-HIV-1 drug).

The antiviral titers are represented as dilutions of herbal extracts at which relative luminescence units were reduced to 50% compared to virus control wells without the test inhibitor. Titers of 100 to 400 were considered to be moderate and titers over 400 to be potent antiviral activities. Ginseng showed moderate antiviral activities against HIV-1 primary isolates of clade B, QH0692.42 and PVO.4. Broadly cross-reactive HIV-1 antiviral activities against all HIV-1 primary isolates tested were consistently observed with Salviae and Cinnamomi extracts. Particularly, Salviae showed potent antiviral activities (> 400) against 4 out of 6 clade B primary isolates and 3 out of 4 clades A, or C isolates. Astragali and Asari extracts were inactive against most primary HIV-1 isolates tested. Also, all herbal samples were tested against a murine retrovirus (SVA-MLV) and showed low activity.

To evaluate the antiviral activities of herbal extracts as a potential candidate for a microbicide against HIV-1, 90% reductions of infectivity were similarly determined (Table 1). The herbal extracts of Salviae and Cinnamomi but not ginseng, Asari, or Astragali could decrease the infectivity of various HIV-1 primary isolates up to 90%. The capacity of these Salviae and Cinnamomi to inactivate 90% of infectious particles indicates a highly potent strength of antiviral activity. The active components in Salviae and/or Cinnamomi may have virucidal activity against clades B, C, and A of HIV-1 isolates, which would be a desirable property for being a promising microbicide and/or antiviral drugs.

Encouraged by the unusually potent anti-HIV-1 activities of Salviae and Cinnamomi herbal extracts, virucidal activities of Salviae and Cinnamomi herbal extracts were further compared with previously demonstrated antiviral compounds including AZT (3'-azido-3'-deoxythymidine) as a positive control. Trichosanthin, a ribosome-inactivating protein extracted from the root tuber of Chinese medicinal herb has been extensively investigated in vitro and clinically as a potential anti-HIV-1 herb, but has some limitations due to its antigenicity, short half-life, and toxicity [15-22]. Dextran sulfate has also been studied for its virucidal activity despite development of resistant virus and low efficacy against HIV-1 R5 primary isolates, and toxicity in a clinical trial [23-26]. Carrageenan, a sulfated polysaccharide, is considered to be a promising microbicide although its safety and efficacy remains to be determined [27-30]. Compared to previously described virucidal compounds, Salviae and Cinnamomi herbal extracts consistently showed similar or higher potency in activity against HIV-1 primary isolate SF162 pseudovirions. It was also reported that aqueous extracts of Chinese medicinal herbs (Prunella vulgaris, Viola yedoensis) inhibited the *in vitro* growth of HIV at a subtoxic concentration [31,32] although they did not inactivate HIV extracellularly (i.e., they were not virucidal) and showed activities only at relatively high concentrations. It is also important to note that non-toxic inhibitors of HIV-1 integrase were isolated from the Salviae extract [33]. HIV-1 integrase inhibitors from

Salviae did not affect the entry and replication of HIV-1, indicating no virucidal activity [33], which is different from our observation of potent virucidal activity of the whole Salviae extract against a broad range of HIV-1 isolates. Therefore, these promising results validate the present study supporting further analysis of the potential active components (Figure 3).

In summary, the results in the present study suggest that Salviae and Cinnamomi have potent anti-HIV-1 virucidal activities. Future studies are needed to identify the active components of the Salviae and Cinnamomi herbal extract, and to better understand the possible action mechanisms of purified components.

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