Polysaccharide from *Aspalathus linearis* with Strong Anti-HIV Activity

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Polysaccharide that had been extracted with 1% sodium carbonate from Rooibos leaves (*Aspalathus linearis*) showed strong anti-HIV activity. Du-Zhong leaves also showed anti-HIV activity, although lower than the extract of *Aspalathus linearis*, but Japanese tea leaves and a hot water extract of *Aspalathus linearis* did not. The anti-HIV activity of the alkaline extract from *Aspalathus linearis* was recovered mainly in the 25–75% ethanol-precipitated fraction. The polysaccharide almost completely inhibited the binding of HIV-1 to MT-4 cells. It is inferred from these results that the polysaccharide from *Aspalathus linearis* is involved in the mechanism for virus binding to T cells.

**Key words:** anti-HIV activity; polysaccharides; in vitro; alkaline extract; *Aspalathus linearis*

It is vitally important to find an effective chemotherapy for the acquired immunodeficiency syndrome (AIDS). Many researchers have been prompted to search for selective antihuman immunodeficiency virus (HIV) agents. Increasing numbers of these anti-viral agents are presently in various stages of development and testing, and some of them have recently been licensed for use by humans. 3'-Azido-3'-deoxythymidine (azidothymidine, AZT) and 2',3'-dideoxynucleosine have improved the clinical and immunological status of patients with AIDS and AIDS-related complex. The target of AZT is viral reverse transcriptase, and the phosphorylated products of AZT would be held responsible for bone marrow suppression. Long-term suppression chemotherapy appears to improve the survival of patients with AIDS, but it is difficult to use for long-term chemotherapy. Its major drug-related toxicity is bone marrow suppression, which limits the dose of AZT that can be used.

There are many substances from plant extracts that have so far shown anti-HIV activity in vitro, including lignin and lignified materials, tannin and related substances, and plant lectins. Unfortunately, these substances showed relatively high cytotoxic activity.

Rooibos tea, a kind of herb tea, which is made of leaves and sprigs of *Aspalathus linearis*, is popular in Republic of South Africa, Europe, and Japan. This plant only grow in Sedarberg mountain, West Cape. Rooibos tea contains a small amount of tannin but no caffeine. The hot-water extract (tea) contains many volatile constituents such as phenolic compounds, many kinds of flavonoids and shows broad pharmacological effects.

We report here that the alkaline extracts from *Aspalathus linearis* strongly suppress the HIV-induced cytotoxic effect with HIV-1 (HTLV-IIB) infected MT-4 cells in vitro, the cytotoxicity being extremely low, and that the substance responsible would be an acid polysaccharide mainly composed of glucose and uronic acid.

**Materials and Methods**

*Materials.* Rooibos tea leaves (*Aspalathus linearis*) and Du-Zhong leaves (*Eucommia ulmoides* Oliv.) were obtained from A.I.C. Co. (Nagoya, Japan) and Nikki Kogyo Co. (Hiroomita, Japan), respectively.

Preparation of the alkaline extract. Unless otherwise indicated, the tea leaves, including sprigs, were extracted with hot water (100-fold volume of the tea leaves) at 85°C for 3 h, and the tea leaves, after having been extracted with hot water, were further extracted twice with 1% sodium carbonate (10-fold the volume of the tea leaves) at 45°C for 3 h. The alkaline extract was filtered through two layers of gauze, and is designated as a “crude extract.”

Ethanol precipitation. The crude extract was fractionated with ethanol in the presence of sodium acetate. Unless otherwise indicated, the extract (1.5 mg/ml) was brought to 0.1 m sodium acetate, 1/4 the volume of ethanol (25% ethanol) was then added, and the mixture was allowed to stand for 1 h at 4°C before being centrifuged at 150 x g for 15 min. An equal volume of alcohol (50% ethanol) was added to the supernatant fraction, and the mixture allowed to stand before being centrifuged as just described. The precipitated fraction is designated as 25–50P. The supernatant fraction obtained was further fractionated with the addition of a three-fold volume of ethanol (75% ethanol), the mixture being allowed to stand for 1 h at 4°C and then centrifuged as described. The final precipitated fraction and supernatant fraction are designated as 50–75P and 75S, respectively. The precipitated fraction between 25% and 75% ethanol is designated as 25–75P.

Column chromatography. The fractions from ethanol precipitation were each dissolved in distilled water, applied to a column (1.6 x 50 cm) of Cellulofine GC-700 m that had been previously equilibrated with distilled water, and then eluted with the same medium.

Cell lines and virus. The human T lymphotropic virus type I (HTLV-I)-positive T cell line, MT-4, and HTLV-I non-infected T cell line, MOLT-4, were grown and maintained in an RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (the complete medium). A strain of HIV-1, HTLV-IIB, was obtained from the culture supernatant of the chronically HIV-1-infected MOLT-4 cells, MOLT-4/HIV-IIB, and stored in a small volume (1 ml) at −80°C until needed. The titer of the virus stock was determined as the 50% cell culture infectious dose (CCID50).

Assay method for anti-HIV activity. Flat-bottom, 96-well plastic microtiter trays (Becton Dickinson, CA, U.S.A.) were filled with 100 μl of the complete medium, and test substances were added. Anti-HIV activity was assayed by the method of Nakashima et al. Briefly, the anti-HIV activity of a test substance was determined by the protection it provided from HIV-induced cytotoxic effect. MT-4 cells were infected with HIV-
1 by 150 CCl₄D₅₀/10⁵ cells. HIV- or mock-infected MT-4 cells (1.5 × 10⁵ cells/ml, 300 µl) were used to evaluate the anti-HIV activity. The HIV-infected MT-4 cells and non-infected (MOLT-4 infected) cells were spread in a 96-well microtiter plate with various concentrations of the test substances, and incubated for 5 days at 37°C in a CO₂ incubator. After this incubation, the number of viable cells was determined by the colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to produce blue-colored formazan. Antivirus activity is expressed as that to give 50% protection of the infected cells by a test substance (EC₅₀, 50% effective concentration). The cytotoxic activity of the test substances is expressed as the cytotoxic concentration of a test substance that resulted in 50% of the cells being damaged (CC₅₀, 50% cytotoxic concentration). The selectivity index (SI) was calculated from the ratio of CC₅₀ to EC₅₀.

Assay for HIV binding. The inhibitory effect of each test substance on the binding of HIV-1 particles to MT-4 cells was determined by an indirect immunofluorescence-laser flow cytometry analysis. Briefly, MT-4 cells were exposed to HIV-1 in the presence or absence of a test substance, the culture being added 10-20 min before adding the virus. The cells were incubated for 60 min at 37°C and washed twice in phosphate-buffered saline (PBS) to remove any unbound virus. A high titer of human polyclonal anti-HIV-1-positive serum was then added. After another 30 min of incubation at room temperature, the cells were washed twice with PBS. The cells were then incubated with the FITC-conjugated rabbit anti-human IgG antibody for 30 min at room temperature, washed twice in PBS, resuspended in 0.37% paraformaldehyde in PBS, and analyzed by laser flow cytometry (CytACE-150; Japan Spectroscopic Co., Tokyo, Japan).

Analytical methods. The contents of neutral sugars and uronic acids were measured by the phenol-sulfuric acid method and the carbazole method, respectively. The composition of neutral sugars was analyzed by HPLC after their digestion with a mixture of trifluoroacetic acid and HCl.

Results

Anti-HIV activity of the alkaline extract

As shown in Fig. 1, the alkaline extract from Aspalathus linearis showed potent anti-HIV activity, which is expressed as the concentration of the extract showing 50% protection of HIV-induced cytopathicity. Thus, the lower the concentration, the higher the activity. We examined the anti-HIV activity of extracts from tea leaves of other cultivars such as Du-Zhong and Japanese (Camellia sinensis var. sinensis). The data are shown in Table I. The activity of the alkaline extract from Aspalathus linearis was 38.9 µg/ml as EC₅₀, 2400 µg/ml as CC₅₀, and 61.7 as SI value (CC₅₀/EC₅₀), these values being the highest of all the extracts tested. Crude hot-water extracts of Aspalathus linearis and alkaline extracts of Japanese tea leaves did not show any anti-HIV activity.

Ethanol fractionation of the crude alkaline extract

The precipitation profile with ethanol was dependent on the concentration of sodium acetate. As shown in Fig. 2, the precipitate (polysaccharide as a neutral sugar) obtained had a moderate precipitation profile in the presence of 0.1 to 0.2 M sodium acetate, at which concentration the active substances were mainly recovered by 25-75% ethanol. With a lower concentration of sodium acetate (0.05 M), an extremely low amount of polysaccharide was obtained between 25% and 50% ethanol, and with a higher concentration of sodium acetate (0.5 M), a high amount of polysaccharide was obtained between 0% and 25% ethanol. Therefore, we used a 0.1 M sodium acetate concentration for the ethanol precipitation. The degree of precipitation by ethanol was also dependent on the concentration of the alkaline extract. The total recovery of the polysaccharide by ethanol precipitation was 40% to 60%.

As shown in Table II, in the case of the 50-75P fraction, EC₅₀ was 8 µg/ml and the SI value was greater than 125. The EC₅₀ and SI values for the 25-75P fraction were 18.3 µg/ml and 54.6, respectively. In the case of the 25-50P

![Fig. 1. Typical Data for the Protection against HIV-induced Cytopathicity by the Alkaline Extract from Aspalathus linearis.](image)

![Fig. 2. Effect of Sodium Acetate Concentration on the Ethanol Precipitation of the Alkaline Extract from Aspalathus linearis.](image)

<table>
<thead>
<tr>
<th>Extracta</th>
<th>Cytotoxicity CC₅₀ (µg/ml)</th>
<th>Anti-HIV activity EC₅₀ (µg/ml)</th>
<th>SIc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rouibos</td>
<td>2400</td>
<td>38.9</td>
<td>61.7</td>
</tr>
<tr>
<td>Du-Zhong</td>
<td>865</td>
<td>169</td>
<td>5</td>
</tr>
<tr>
<td>JPN</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RBT</td>
<td>750</td>
<td>950</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a Rouibos, crude alkaline extract from Aspalathus linearis; Du-Zhong, crude alkaline extract from Eucommia ulmoides Oliv.; JPN, crude alkaline extract from Japanese tea leaves (Camellia sinensis); RBT, crude hot-water extract from Aspalathus linearis.

b-c CC₅₀, EC₅₀, and SI values are described in Materials and Methods.
fraction, the EC$_{50}$ and SI values were almost the same as those of the 25–75P fraction, which were about 2-fold higher than those of the crude extract. However, the 75S fraction did not show any anti-HIV activity.

Column chromatography of the ethanol-precipitated fraction
The active fraction after ethanol precipitation (25–75P) was applied to a column of Cellulofine GC-700 m. Figure 3 shows the chromatographic profile in the Cellulose GC-700 m column of ethanol-precipitated fraction 25–75P from *Aspalathus linearis*. There were two peaks, the main one at the void volume and a minor peak in a low molecular weight region (fraction No. 38). Both fractions 25–50P and 50–75P showed a similar pattern to that of fraction 25–75P (data not shown). We used fraction 25–75P for the subsequent experiments, because it contained a larger amount of the active substances.

Sugar composition of the active fraction
As can be seen in Table III, the purified polysaccharide (fraction 25–75P) from *Aspalathus linearis* was composed

<table>
<thead>
<tr>
<th>Sugar component</th>
<th>Content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>63.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>10.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>16.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>10.6</td>
</tr>
</tbody>
</table>

Table III. Sugar Components of the Purified Polysaccharide from *Aspalathus linearis*

Table II. Effect of Ethanol Precipitation of the Alkaline Extract from *Aspalathus linearis*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytotoxicity CC$_{50}$ (µg/ml)</th>
<th>Anti-HIV activity EC$_{50}$ (µg/ml)</th>
<th>SI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (n=6)</td>
<td>&gt;1000</td>
<td>42.6</td>
<td>&gt;23.5</td>
</tr>
<tr>
<td>25–75P (n=5)</td>
<td>&gt;1000</td>
<td>18.3</td>
<td>&gt;54.6</td>
</tr>
<tr>
<td>25–50P (n=3)</td>
<td>&gt;1000</td>
<td>22.5</td>
<td>&gt;44.6</td>
</tr>
<tr>
<td>50–75P (n=3)</td>
<td>&gt;1000</td>
<td>8.0</td>
<td>&gt;125</td>
</tr>
<tr>
<td>75S (n=5)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

$^c$ CC$_{50}$, EC$_{50}$, and SI values are described in Materials and Methods.

Table III. Sugar Contents of the Alkaline Extracts from *Aspalathus linearis* and *Eucommia ulmoides* Oliv.

<table>
<thead>
<tr>
<th>Component</th>
<th>Du-Zhong (mg/100 mg)</th>
<th>Rooibos (mg/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugars</td>
<td>6.33 ± 0.525</td>
<td>26.3 ± 1.69</td>
</tr>
<tr>
<td>Neutral sugars</td>
<td>17.3 ± 0.690</td>
<td>48.9 ± 16.82</td>
</tr>
<tr>
<td>Uronic acid (A)</td>
<td>10.1 ± 0.797</td>
<td>15.4 ± 1.94</td>
</tr>
<tr>
<td>Uronic acid (B)</td>
<td>16.3 ± 1.34</td>
<td>24.9 ± 3.25</td>
</tr>
</tbody>
</table>

Each value shows the mean ± SD. Rooibos, alkaline extract from *Aspalathus linearis*; Du-Zhong, alkaline extract from *Eucommia ulmoides* Oliv. Uronic acids were determined by the carbazole method, using glucuronic acid (A) and galacturonic acid (B) as the standards.
of 26.3% reducing sugar, 48.9% neutral sugars, and 15.4–24.9% uronic acid. In the case of Du-Zhong, the composition of the polysaccharide was 6.3% of reducing sugar, 17.3% of neutral sugars, and 10.1–16.3% of uronic acid. We next analyzed the neutral sugar composition of the polysaccharide from *Aspalathus linearis*. As shown in Table IV, the polysaccharide was mainly composed of glucose (63.8 mol%), and the other components being mannose (16.3 mol%), galactose (10.2 mol%), and xylose (10.6 mol%) as the neutral sugars.

**Protection of HIV binding to MT-4 cells**

We examined the effect of the polysaccharide on the mechanism for viral replication. As can be seen in Fig. 4, the polysaccharide (fraction 25–75P) from *Aspalathus linearis* almost completely blocked the binding of HIV to MT-4 cells at a concentration of 250 μg/ml. The fluorescence of cells that had not been exposed to HIV-1 served as a control (non-specific fluorescence; unshaded histogram). The shaded histograms represent cellular fluorescence resulting from the specific binding of the HIV-1 antibody to MT-4 cells which had adsorbed the HIV-1 virion (Fig. 4A). The mean fluorescence values for the MT-4 cells that had been exposed to HIV-1 decreased to the background (control) level when the cells had been preincubated with dextran sulfate (Fig. 4B) or with the polysaccharide from *Aspalathus linearis* (Fig. 4C). Dextran sulfate was used as a positive control for the HIV-binding experiment.

**Discussion**

Since the discovery of HIV as the causative agent of AIDS, various chemotherapeutic drugs have been tested to control this fatal disease. HIV is perhaps the most complex retrovirus studied, offering a variety of potential points of attack such as the inhibition of viral binding, reverse transcriptase inhibitors, and protease inhibitors. Several antiviral drugs have so far been developed. 1–5 Although AZT and 2,3'-dideoxynosine are available for treating AIDS and AIDS-related complex, these agents have shown severe side effects with long-term chemotherapy. 6–7 Therefore, it is extremely important to find effective substances without side effects. The present paper reports that an alkaline extract from *Aspalathus linearis* had strong anti-HIV activity with extremely low cytotoxicity (Fig. 1), the 50% cytotoxic concentration (CC₅₀) being 2.4 mg/ml (Table I).

The crude water extract of *Aspalathus linearis* contained a large amount of flavonoids and polyphenolic substances. 10–12 The alkaline extract from *Aspalathus linearis* was mainly recovered in fraction 25–75P by ethanol precipitation (Table II), and the extract contained a larger amount of neutral sugars and uronic acids than that from *Eucommia ulmoides* Oliv. (Table III). These polysaccharides could not detect proteins, so they would have been acid polysaccharides, even though the contents of uronic acid would be different between *Aspalathus linearis* and *Eucommia ulmoides* Oliv. Moreover, the content of active substances in the plants would be different from species to species, and according to extraction procedure.

Generally, the life-cycle of a retro-virus firstly involves virus attachment to the target cells, before virus-induced cell fusion (syncytium formation) occurs, and then reverse transcription, formation of a provirus and new virus replication proceed in the host cell. The infection of T-lymphocytes and macrophages by HIV is mediated by the binding of the HIV envelope glycoprotein (gp120) to cell-surface receptor glycoprotein CD4, which is an integral membrane glycoprotein of CD4⁺ cells. 23–25 High-molecular-weight substances from plants 16,26–28 and sulfated polysaccharides 26,29 such as dextran sulfate, heparin, and pentosan polysulfate have shown anti-HIV activity. It is suggested that these substances are involved in the mechanism of virus binding to the cells. As shown in Fig. 4, the polysaccharide from *Aspalathus linearis* almost completely blocked virus binding to the cells at a concentration of 250 μg/ml. Thus, the polysaccharide described in this paper would be involved in the mechanism of virus binding to the cells, the initial step in the replication of HIV.

The first step in the infection of cells by HIV is attachment of the virions to the conserved region of the CD4 antigen, where binding involves gp120-CD4 interaction. Substances that inhibit viral binding would be superior to the drugs associated with other stages of HIV replication. If a substance can block viral binding to the cells, the most complex retrovirus, HIV, would never be able to penetrate into the cells. In this case, it could be easier to control viral replication. Sulfated polysaccharides such as dextran sulfate and pentosan sulfate block not only virus binding but also giant cell formation (syncytium) in vitro. However, none of these compounds has been found to have activity in clinical use on patients with HIV infection. 30 The polysaccharide described in this paper showed no cytotoxic effect at a concentration lower than 2.4 mg/ml, and showed anti-HIV activity at a relatively low concentration. Even though the exact mechanism by which this reported polysaccharide blocks viron binding to the cells remains unknown, it is likely that this substance is a good candidate drug and will add a new dimension to the therapeutic weapons against AIDS.

**References**

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