Inhibition of HIV-1 Reverse Transcriptase Activity by the Extracts of Indian Plants

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As the currently available therapies for HIV-1 have high costs and are associated with the appearance of multi-drug resistant HIV-1 strains, the Indian traditional medicine system Ayurveda may provide useful alternatives. In this study, we screened the inhibitory activities of various extracts of five Indian plants for inhibitory effects on HIV-1 reverse transcriptase (RT). Water extracts of the leaves of Argemone mexicana strongly inhibited the DNA polymerase activity of HIV-1 RT, which indicated that they contained substances with inhibitor activity. Neither heat treatment at 100°C nor proteinase K treatment of the extracts abolished the inhibitory activity, suggesting that the inhibitory substance was an organic compound rather than a protein.

Key words: Argemone mexicana; HIV-1; inhibition; Parthenium hysterophorus; reverse transcriptase.

Introduction
Reverse transcriptase (RT) [EC 2.7.7.49] from human immunodeficiency virus type 1 (HIV-1) possesses RNA- and DNA-dependent DNA polymerase activities and RNase H activity. It is a heterodimer consisting of a 66-kDa p66 subunit and a 51-kDa p51 subunit. The former includes the fingers, palm, thumb, and connection subdomains and the RNase H domain, while the latter lacks the RNase H domain and has the other four subdomains. The fingers, palm, and thumb subdomains are involved in the DNA polymerase activity, and the RNase H domain is in the RNase H activity.

In the current HIV-1 therapy, nucleoside/nontenucleoside RT inhibitors, protease inhibitors, and integrase inhibitors are used,
which is highly expensive. In addition, the long-term use of such inhibitors has caused the emergence of multi-drug resistant HIV-1 strains, which is a major issue. Therefore, the development of a novel and cheap HIV-1 RT inhibitor is anticipated.

We previously screened the inhibitory activities of 25 edible plants against the DNA polymerase activity of HIV-1 RT and found that the ethanol and water extracts of *Brasenia schreberi* (Junsai) exhibited strong inhibitory activities [1, 2]. Purification and structural determination of the constituent compounds are currently in progress.

Ayurvedic medicine is an Indian traditional medicine system, which offers inexpensive, natural, orally prescribed remedies of extracts of the leaves, flowers, and fruits of plants. However, little is known about the application of Ayurvedic medicine to HIV-1 therapy. In this study, we selected five Indian plants whose extracts were available in University of Allahabad as research targets: *Parthenium hysterophorus* (Gajar Ghas), *Argemone mexicana* (Satyanashi), *Solanum xanthocarpum* (Kantakari), *Calotropis procera* (Safed aak), and *Thevetia peruviana* (Peeli Kancer). *P. hysterophorus* is an annual weed utilized as an ethnomedicine for the treatment of different infectious and chronic diseases. In addition, extracts of *P. hysterophorus* have been found to be useful for the treatment of anemia, fever, wound healing, heart troubles, and sores. In contrast, the plant has the ability to kill other plants growing in its vicinity, and causes allergic diseases in humans and animals [3, 4]. It is found in almost all parts of the world. *A. mexicana* is a species of poppy. Despite being poisonous, all parts of this plant have been used in traditional medicine to treat chronic diseases of the skin, gastrointestinal tract, and respiratory tract. In addition, it exhibits allelopathic, larvicidal, nematicidal [5], and antimicrobial potential, in addition to chemosterilant and wound-healing activities [6]. It is found in Ethiopia, Mexico, United States, and many warm places in India. *S. xanthocarpum* is a species of herb. It is a medicinal plant that is used mostly in India. Some parts of the plant, especially the fruit, are poisonous [7]. *C. procera* is a species of flowering plant. The milky sap contains a complex mix of chemicals, some of which are steroidal heart poisons [8]. *T. peruviana* is an evergreen shrub or small tree. The plant is used widely in folk medicine. In India, it has become established as a household remedy for several diseases [9].

In this study, we screened for HIV-1 RT inhibitory activity in these five Indian plants. The results showed that the water extracts of the leaves of *A. mexicana* strongly inhibited HIV-1 RT activity.

**Materials and Methods**

**Materials**  
pd(T)_{15} was purchased from Fasmac (Tokyo, Japan). [methyl-^3^H]dITTP (1.52 TBq/mmol) and poly(rA) were from GE Healthcare (Buckinghamshire, UK). Recombinant HIV-1 RT was expressed in *Escherichia coli* and purified from the cells as described previously [10]. The RT concentration was determined using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as standard.

**Preparation of extracts of Indian plants**  
Freeze-dried leaves, flowers, and fruits were crushed into a powdered form by using a blender. The powders were then extracted with water, methanol, ethyl acetate, or hexane at room temperature (20-25°C) or at higher temperatures, equivalent to the boiling point of the respective solvents, by using a Soxhlet apparatus. The solution of the extracts thus prepared were evaporated to dryness at room temperature. The
dried extracts were dissolved in the required volume of the corresponding solvent.

Measurement of HIV-1 RT activity using \[^{3}H\]-dTTP  HIV-1 RT was incubated with extracts: 80 \(\mu\)l of the HIV-1 RT solution (55 nM in 20 mM potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol (DTT), 10% v/v glycerol (buffer A)) and 32 \(\mu\)l of extract were mixed and incubated at 37°C for 5 min. Then, the activity was measured as described previously [11]. Briefly, the reaction mixture (52 \(\mu\)l) was carried out in 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM DTT, 5.0 mM MgCl\(_2\), 25 \(\mu\)M poly(rA)-<br>poly(dT)\(_{16}\) (this concentration is expressed as that of poly(dT)\(_{16}\), 0.2 mM \[^{3}H\]-dTTP, 5 nM HIV-1 RT, and 8% v/v extract at 37°C. An aliquot (20 \(\mu\)l) was taken from the reaction mixture at a predetermined time and immediately spotted onto the glass filterGF/C 2.5 cm (Whatman, Middlesex, UK). The amounts of \[^{3}H\]-dTTP incorporated was counted, and the reaction rate was determined.

Measurement of HIV-1 RT activity using fluorescent dye PicoGreen  HIV-1 RT was incubated with extracts as described above. Then, the activity was measured with EnzChek Reverse Transcriptase Assay Kit (Thermo Fisher Scientific, Waltham, MA) as described previously [11]. Briefly, PicoGreen solution was prepared by 200-fold diluting PicoGreen dsDNA quantification reagent with 1×TE buffer (10 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA). Poly(rA)-<br>poly(dT)\(_{16}\) solution was prepared by mixing poly(rA) (5 \(\mu\)l) and poly(dT)\(_{16}\) (5 \(\mu\)l) and incubating for 1 h at room temperature followed by the dilution with 2 ml of polymerization buffer (60

(A)

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant name</th>
<th>Part of plant</th>
<th>Solvent</th>
<th>Temperature at extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Leaf</td>
<td>Water</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Leaf</td>
<td>Ethyl acetate</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>Parthenium hysterosorus</td>
<td>Leaf</td>
<td>Hexane</td>
<td>H</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Flower</td>
<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Flower</td>
<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Flower</td>
<td>Methanol</td>
<td>H</td>
</tr>
<tr>
<td>7</td>
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<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Leaf</td>
<td>Methanol</td>
<td>R</td>
</tr>
<tr>
<td>9</td>
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<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
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<td></td>
<td>Leaf</td>
<td>Water</td>
<td>H</td>
</tr>
<tr>
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<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>Fruit</td>
<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
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<td>Hexane</td>
<td>H</td>
</tr>
<tr>
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<td>Solanum xanthocarpum</td>
<td>Fruit</td>
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</tr>
<tr>
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<td></td>
<td>Fruit</td>
<td>Ethyl acetate</td>
<td>H</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>Fruit</td>
<td>Water</td>
<td>R</td>
</tr>
<tr>
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<td>Ethyl acetate</td>
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</tr>
<tr>
<td>19</td>
<td></td>
<td>Flower</td>
<td>Water</td>
<td>H</td>
</tr>
<tr>
<td>20</td>
<td>Thevetia peruviana</td>
<td>Fruit</td>
<td>Water</td>
<td>H</td>
</tr>
</tbody>
</table>

(B)

Fig. 1. Inhibition of HIV-1 RT activity by 20 extracts. (A) Origin and preparation method of the extracts. R and H indicates room temperature and temperatures equivalent to the boiling point of the respective solvents, respectively. (B) HIV-1 RT activity in the presence of extracts. The results of the assay using \[^{3}H\]-dTTP and that using fluorescent dye PicoGreen are shown. The reaction was carried out at 37°C with HIV-1 RT at 5 nM and extracts at 8% v/v. The reaction without extracts was also carried out as a control. Relative activity was defined as the ratio of the reaction rate with extracts to that without it. One of the representative data of the average of triplicate determinations with SD value is shown.
mM Tris-HCl buffer (pH 8.1), 60 mM KCl, 8 mM MgCl₂, 13 mM DTT, 100 µM dTTP). The reaction was started by adding the pre-incubated solution of HIV-1 RT and extract (24 µl) to the poly(rA)-p(dT)₁₆ solution (96 µl). An aliquot (25 µl) was taken from the reaction mixture at 2.5, 5.0, and 7.5 min, to which 2 µl of 200 mM EDTA was immediately added and incubated on ice for 30 min. Blank solution was prepared by mixing poly(rA)-p(dT)₁₆ solution (20 µl), 200 mM EDTA (2 µl), and HIV-1 RT solution (5 µl). To each solution (27 µl), PicoGreen solution (173 µl) was added. The tubes were wrapped with aluminum foil and left at room temperature for 10 min. The fluorescence at 523 nm was measured with EnSight (PerkinElmer, Waltham, MA) with the excitation wavelength of 502 nm.

Results and Discussion

Screening of HIV-1 RT inhibitory activity

We tested the inhibitory activities of 20 extracts on HIV-1 RT by using [³H]-dTTP or the fluorescent dye PicoGreen (Fig. 1). The relative activity was defined as the ratio of the reaction rate with 8% v/v extract to that without it. The water extracts of the leaves of *P. hysterophorus* (No. 1) and *A. mexicana* (No. 11) inhibited HIV-1 RT activity by more than 90%, whereas the ethyl acetate, hexane, and methanol extracts inhibited HIV-1 RT activity by 17%-58%. The difference in inhibitory activity between water extracts and other extracts was probably due to the different constituents that were extracted by water and other solvents. The extracts of the leaves, fruits, and flowers of *S. xanthocarpum*, *C. procera*, and *T. peruviana* inhibited HIV-1 RT activity by 10%-80%.

The water extract of the leaves of *P. hysterophorus* (No. 1) and water extract of the leaves of *A. mexicana* (No. 11) showed the strongest inhibitory activity. Thus, these two

![Fig. 2. Inhibition of HIV-1 RT by water-extracts of the leaf of *P. hysterophorus* (circle) and *A. mexicana* (triangle), and water extracts of *B. schreberi* (square). The results of the assay using [³H]-dTTP are shown. Relative activity was defined as the ratio of the reaction rate with extracts to that without it. One of the representative data of the average of triplicate determinations with SD value is shown.](image)

![Fig. 3. Effects of thermal treatment and protease treatment on the inhibitory activity. The results of the assay using [³H]-dTTP is shown. Relative activity was defined as the ratio of the reaction rate with extracts to that without it. The concentrations of the extracts of *A. mexicana* and *B. schreberi* were 100 and 400 µg/ml, respectively, in the thermal treatment at 100°C for 5 min, 160 and 400 µg/ml, respectively, in the protease treatment (50 µg/ml in 20 mM Tris-HCl buffer (pH 7.5) at 37°C for 60 min), and 64 and 160 µg/ml, respectively, in the RT reaction. One of the representative data of the average of duplicate determinations are shown.](image)
extracts were subjected to further analysis.

HIV-1 RT inhibitory activity of water extracts of the leaves of P. hysterophorus and A. mexicana

We examined the effect of increasing concentrations of water extracts of the leaves of P. hysterophorus and A. mexicana on HIV-1 RT activity (Fig. 2). B. schreberi (Junsai) was also examined as a comparator. The reaction rates decreased as the concentrations of the extracts increased. The relative activity was 20% at 160 μg/ml for P. hysterophorus, 0% at 240 μg/ml for A. mexicana, and 0% at 80 μg/ml for B. schreberi. The concentrations resulting in 50% effective inhibition (IC₅₀ values) were estimated to be 60.4 μg/ml for P. hysterophorus, 23.7 μg/ml for A. mexicana, and 50.2 μg/ml for B. schreberi. These results indicated that the inhibitory effects of P. hysterophorus and A. mexicana were equally strong. As IC₅₀ values of various plants extracts used in traditional medicine against the DNA polymerase activity of HIV-1 RT were in the range of 2 to 100 μg/ml [12], the IC₅₀ values of A. mexicana and P. hysterophorus were high enough.

Effects of thermal treatment and proteinase treatment on the inhibitory activity.

To determine whether the inhibitory substances of the leaves of A. mexicana was an organic compound or a protein, we examined the inhibitory activity of the extracts that had been subjected to heat treatment (100°C for 10 min) or proteinase K treatment (Fig. 3). Neither thermal treatment nor proteinase K treatment abolished the inhibitory activity of the leaves. The same results were obtained for the water extracts of B. schreberi.

Various enzyme inhibitors have been identified in natural product. In the case of HIV-1 RT, 5,6,7-trihydroxyflavone (baicalein) [13] and catechins containing a galloyl moiety such as (−)-epicatechin 3-gallate (ECG) [14] and (−)-epigallocatechin 3-gallate (EGCG) [15] were reported to inhibit the DNA polymerase activity of HIV-1 RT. However, India has numerous plant resources, and our results have suggested that there may be as yet unidentified Indian plants that possess strong HIV-1 RT inhibitory activity. In combination with Ayurvedic techniques, these plants may be developed as a novel, inexpensive pharmaceutical agent for HIV-1 therapy.

References

Inhibition of HIV-1 Reverse Transcriptase


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