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Article

Inhibition of HIV-1 Reverse Transcriptase Activity by *Brasenia schreberi* (Junsai) Components

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We screened for inhibitory activities of 25 wild vegetables and fruits for human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Among them, ethanol- and water-extracts of *Brasenia schreberi* (Junsai) and water-extract of *Petasites japonicus* (Fuki) strongly inhibited the HIV-1 RT activity to incorporate dTTP into poly(rA)-p(dT)$_{15}$. We tested HIV-1 RT inhibitory activities of 15 polyphenols, isolated from *Brasenia schreberi*. Among them, gossypetin and hypolaetin 7-*O*-glucoside inhibited the activity.

Key words: *Brasenia schreberi*, HIV-1; inhibition, reverse transcriptase.

Introduction

Reverse transcriptase (RT) [EC 2.7.7.49] is the enzyme responsible for replication of retrovirus. It possesses RNA- and DNA-dependent DNA polymerase activities and RNase H activity. Human immunodeficiency virus type 1 (HIV-1) RT is a heterodimer consisting of a 66-kDa p66 subunit and a 51-kDa p51 subunit [1, 2]. In the HIV-1 RT therapy, nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) are used. However, the emergence of RT inhibitor-resistant HIV-1 variants is a major issue. Therefore, the development of a novel HIV-1 RT inhibitor is anticipated.

Various enzyme inhibitors have been identified in natural product. In the case with HIV-1 RT, 5,6,7-trihydroxyflavone (baicalein) [3] and catechins containing a galloyl moiety such as (-)-epicatechin 3-gallate (ECG) [4] and (-)-epigallocatechin 3-gallate (EGCG) [5] were reported to inhibit the RNA-dependent DNA polymerase (reverse transcriptase) activity of HIV-1 RT. However, it was pointed out that their inhibitory activities were non-specific, due to their binding abilities to various proteins [6]. Indeed, it was recently reported that methyl gallate, purified from *Pholiota adipose* (Mushroom), completely inhibited HIV-1 RT activity [7].

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We previously prepared recombinant RTs from HIV-1 group M, spread all over the world, and HIV-1 group O, restricted to Cameroon and neighboring countries in West Central Africa, and compared their activities and stabilities [8, 9]. In the present study, we screened for HIV-1 RT inhibitory activity in various foodstuffs using the HIV-1 group M RT and found the strong inhibitory activity in ethanol- and water-extracts of *Brasenia schreberi* (Junsai).

Materials and Methods

Materials $p(dT)_{15}$ was purchased from Life Technologies Japan Ltd. (Tokyo, Japan). [methyl-^{3}H]dTTP (1.52 TBq/mmol) and poly(rA) were from GE Healthcare (Buckinghamshire, UK). Recombinant HIV-1 group M RT was expressed in *Escherichia coli* and purified from the cells as described previously [8, 9]. 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 foodstuffs Freeze-dried wild vegetables and fruits were reduced to powder. In the preparation of ethanol-extracts, the powder was extracted with ethanol and evaporated to dryness. The evaporated sample was dissolved in ethanol at a final concentration of 10-20 mg/ml. In the preparation of water-extracts, the powder was added to water and autoclaved at 105ºC for 30 min. After centrifugation, the supernatant were evaporated to dryness. The dry matter was dissolved in water at a final concentration of 10-40 mg/ml.

Measurement of HIV-1 RT activity RT activity for incorporation of dTTP into poly(rA)-$p(dT)_{15}$ was measured as described previously [8, 9]. In the experiment with extracts of foodstuffs, pre-incubation (20 μl) was initiated by mixing 18 μl of the HIV-1 RT solution (220 nM in 20 mM potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol (DTT), 10% v/v glycerol (buffer A)) and 2 μl of ethanol- or water-extract of foodstuffs. In the experiment with compounds, pre-incubation (20 μl) was initiated by mixing 18 μl of the HIV-1 RT solution (220 nM in buffer A) and 2 μl of the compound solution (0−110 μM in dimethyl sulfoxide (DMSO)). After the pre-incubation at room temperature for 3 min, the reaction was carried out in 25 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2.0 mM DTT, 5.0 mM $MgCl_2$, 25 μM poly(rA)-$p(dT)_{15}$ (this concentration is expressed as that of $p(dT)_{15}$), 0.2 mM [^{3}H]dTTP, 2 μM HIV-1 RT, and 1% v/v extract or 0−10 μM compound at 37ºC. An aliquot (20 μl) was taken from the reaction mixture at a predetermined time and immediately spotted onto the glass filter GF/C 2.5 cm (Whatman, Middlesex, UK). The amounts of [^{3}H]dTTP incorporated was counted in 2.5 ml of Ecoscint H (National Diagnostics, Atlanta, GA) with a liquid scintillation counter LSC-5100 (Aloka, Mitaka, Japan), and the reaction rate was determined.

Results and Discussion

HIV-1 RT inhibitory activities of extracts of foodstuffs

We tested inhibitory activities of ethanol- and water-extracts of 25 wild vegetables or fruits for HIV-1 RT to incorporate dTTP into poly(rA)-$p(dT)_{15}$ (Fig. 1). The relative activity

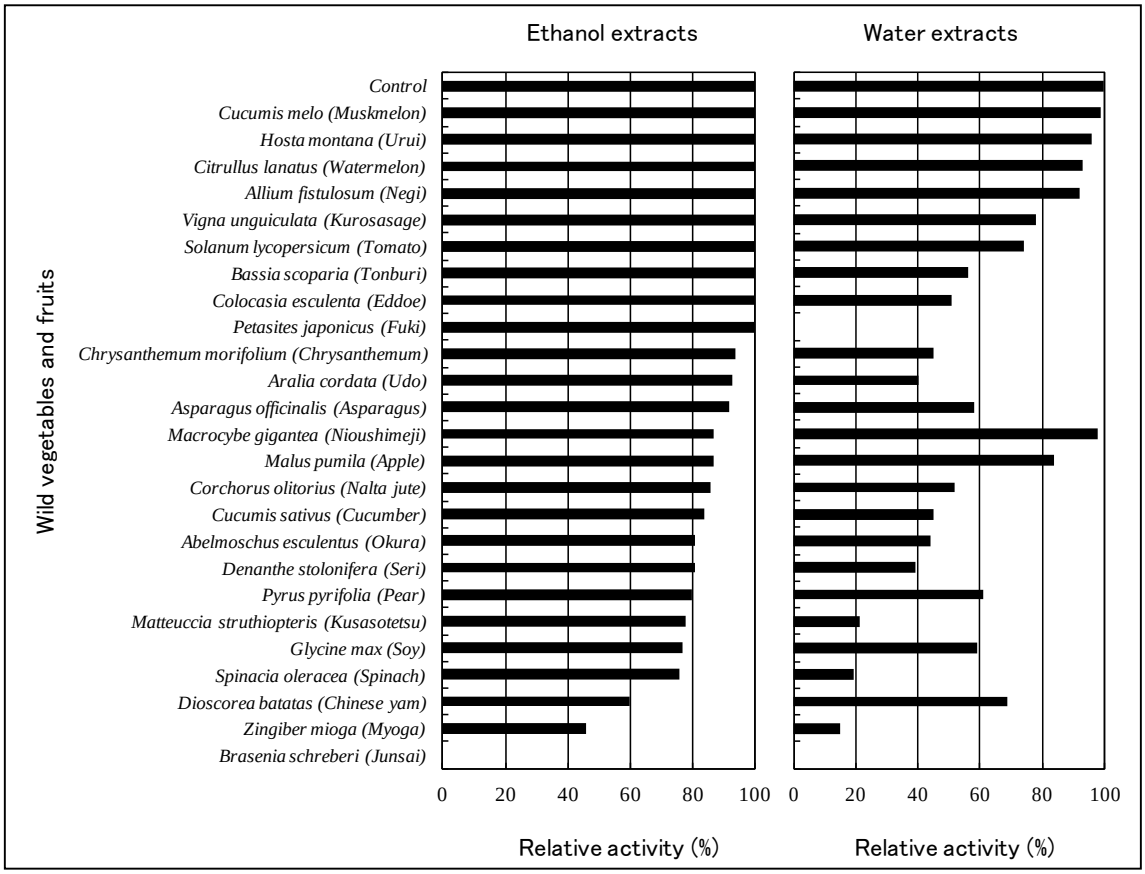

Fig. 1. Inhibition of HIV-1 RT activity by extracts of 25 wild vegetables or fruits.

The reaction was carried out at 37°C with HIV-1 RT at 20 nM and ethanol- or water-extracts of wild vegetables and fruits at 1% v/v. Control means the reaction with 1% ethanol instead of ethanol-extracts in left panel and that with 1% water instead of water-extracts in right panel. The relative activity was defined as the ratio of the reaction rate with extracts to that of control (43.6×10^{-9} M s^{-1} in left panel and 56.3×10^{-9} M s^{-1} in right panel).

was defined as the ratio of the reaction rate with 1% v/v extract to that without it. Among them, ethanol- and water-extracts of *Brasenia schreberi* (Junsai) and water-extract of *Petasites japonicus* (Fuki) completely inhibited HIV-1 RT activity. In all foodstuffs except for *Macrocybe gigantea* (Nioushimeji) and *Dioscorea batatas* (Chinese yam), water-extract exhibited stronger inhibitory activities than ethanol- extract.

We compared the inhibitory activities of ethanol- and water-extracts of *Brasenia schreberi* and water-extract of *Petasites japonicus*. At 0.1% v/v, ethanol- and water-extracts of *Brasenia schreberi* and water-extract of *Petasites japonicus* inhibited HIV-1 RT activity by 32, 6, and 36%, respectively, compared with control (data not shown), indicating that water-extract of *Brasenia schreberi* had the strongest inhibitory activity.

HIV-1 RT inhibitory activities of compounds isolated from *Brasenia schreberi*

Brasenia schreberi is an aquatic plant which lives in pure water. Gallic acid, quercetin, and quercetin 7-*O*-glucoside were previously reported to be isolated from leaves of *Brasenia schreberi* produced in Canada [10]. Recently, 15

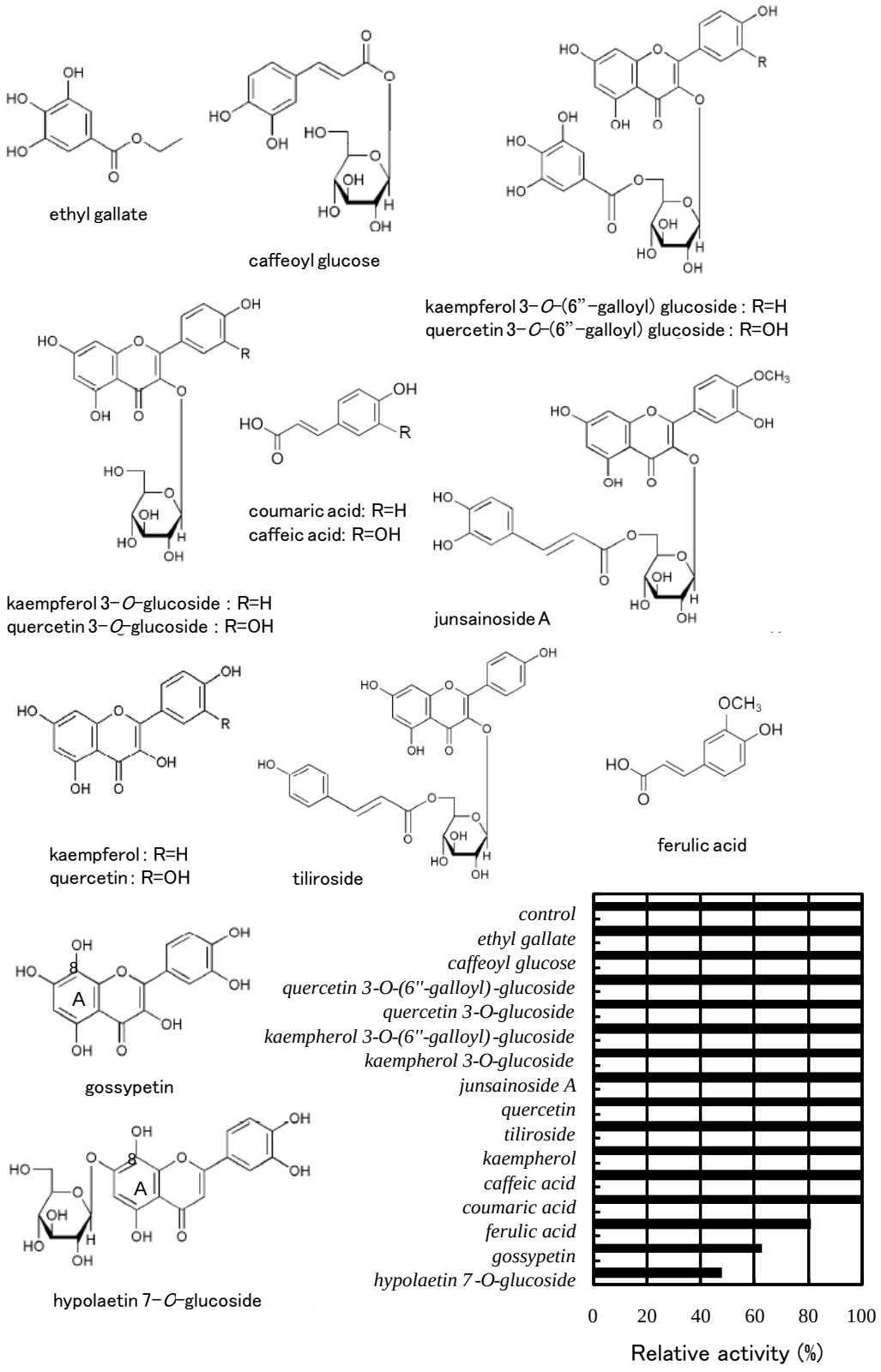

Fig. 2. Inhibition of HIV-1 RT activity by 15 compounds isolated from *Brasenia schreberi*

The reaction was carried out in 10% DMSO at 37°C with HIV-1 RT at 20 nM and compounds at 10 μM. Control means the reaction without compounds. The relative activity was defined as the ratio of the reaction rate with compound to that without it (49.5×10^{-9} M s^{-1}).

compounds were isolated from leaves of *Brasenia schreberi* produced in Akita, Japan (J. T., H. S., unpublished results) (Fig. 2). They include a new compound, named junsainoside A. We tested inhibitory activities of these 15 compounds for HIV-1 RT (Fig. 2). The relative activity was defined as the ratio of the reaction rate with compound to that without it. Among them, hypolaetin 7-*O*-glucoside, gossypetin, and ferulic acid inhibited HIV-1 RT activity by 52, 38, and 19%, respectively, compared with control, while other 12 compounds did not inhibit it. Hypolaetin 7-*O*-glucoside and gossypetin, unlike other 13 compounds, have the hydroxyl group at the 8 position of the A ring (Fig. 2), although it is unknown if it is responsible for the inhibition.

Ethanol- and water-extracts of *Brasenia schreberi* inhibited the activity completely (Fig. 1), while hypolaetin 7-*O*-glucoside or gossypetin did not inhibit it completely (Fig. 2). It was reported that gossypetin [11] and quercetin [12] inhibited the HIV-1 RT activity. The IC_{50} values of quercetin was 60 μM [12], while in this study, quercetin at 10 μM did not exhibit the inhibitory activity (Fig. 2). As far as we know, there has been no report of inhibition of HIV-1 RT by hypolaetin 7-*O*-glucoside. In regard of this, it was reported that hypolaetin, kaempferol, and quercetin inhibited the HIV-1 integrase activity [13].

Manner of inhibition of gossypetin in HIV-1 RT activity

We measured the reaction rates for the incorporation of dTTP into poly(rA)-p$(dT)_{15}$, in the presence and the absence of various concentrations of gossypetin. All the plots showed saturated profiles (Fig. 3A). The plot of [dTTP]/v_o *vs.* [dTTP] (Hanes-Woolf plot) in the presence and the absence of gossypetin showed non-parallel lines intersecting at the Y-axis, indicating that inhibition was uncompetitive (Fig. 3B). The apparent K_m values ($K_{m,app}$) observed for 0, 1.0, 2.5, 5.0, and 10 μM gossypetin were determined to be 40 ± 8, 31 ± 4, 24 ± 4, 10 ± 2, and 13 ± 2 μM, respectively, and the apparent k_{cat} values ($k_{cat,app}$) observed for 0, 1.0, 2.5, 5.0, and 10 μM gossypetin were determined to

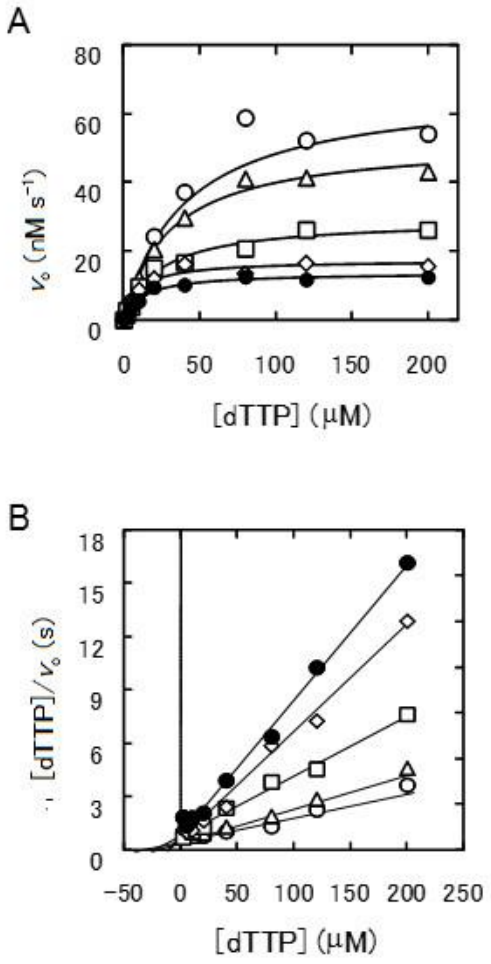

Fig. 3. Dependence on the substrate concentration of the reaction rate of HIV-1 RT-catalyzed reverse transcription in the presence of gossypetin.

The reaction was carried out in 10% DMSO with HIV-1 RT at 20 nM in the presence of 0 (open circle), 1.0 (open triangle), 2.5 (open square), 5.0 (open diamond), and 10 μM (solid circle) gossypetin at 37°C. (A) Effect of the initial dTTP concentrations, $[dTTP]_o$, on reaction rate, v_o. Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. (B) Hanes-Woolf plot.

be 3.4 ± 0.2, 2.6 ± 0.1, 1.5 ± 0.1, 0.86 ± 0.05, and 0.68 ± 0.03 s^{-1}, respectively. Based on this, the reaction rate can be described by the following equation:

$$v_o = \frac{k_{cat,app}[E]_o[S]}{K_{m,app} + [S]} = \frac{k_{cat} / \left(1 + \frac{[I]_o}{K_i}\right) [E]_o [S]}{K_m / \left(1 + \frac{[I]_o}{K_i}\right) + [S]}$$

where $[I]_o$ and K_i are the initial inhibitor concentration and the inhibitor constant respectively. The K_i value of gossypetin was calculated to be 1.5 ± 0.7 μM from the equation. As for hypolaetin 7-*O*-glucoside, we did not examine the manner of inhibition because we did not obtain an adequate amount of samples.

Various enzyme inhibitory activities were found in foodstuffs, from some of which inhibitors were purified and identified. In the case with renin inhibitor, inhibitory activities were found in soybean [14], rice [15], and *Aralia cordata* (Udo) [16], from each of which soyasaponin I [14], oleic acid and linoleic acid [15], and kaurenic acid and pimaradienoic acids [16] were identified as renin inhibitor, respectively. In this study, ethanol- and water-extracts of *Brasenia schreberi* inhibited the activity completely while isolated compounds did not inhibit it completely.

Recently, Takahashi *et al.* screened for inhibitory activities of seven wild vegetables for antihyperlipidemic activities and found that ethanol- and water-extracts of *Brasenia schreberi* exhibited strong inhibitory activities against triglyceride and cholesterol secretions from human hepatoma cells [15]. Purification and identification of the inhibitory compound has not been reported.

In conclusion, *Brasenia schreberi* might be an important source of drugs. The purification and identification of the HIV-1 RT inhibitory compound from *Brasenia schreberi* is a next research subject.

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