JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 1275–1280

Received March 8, 2006
Accepted May 26, 2006
DOI 10.1211/jpp.58.9.0015
ISSN 0022-3573

Compounds from rose (*Rosa rugosa*) flowers with human immunodeficiency virus type 1 reverse transcriptase inhibitory activity

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Abstract

The aqueous extracts and ethanol precipitates of aqueous extracts of 18 medicinal herbs traditionally used in China were screened for their ability to inhibit human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT) in-vitro. Among the samples screened at a concentration of 500 μg mL^{-1}, dried rose (*Rosa rugosa*) flowers showed the strongest inhibition. The ethanol precipitate of the aqueous extract of *R. rugosa* was processed and two components (P_1 and P_2) were obtained after ion exchange chromatography on DEAE-cellulose. Then, $P_{1\text{-a}}$ (Mr 150 kDa) and $P_{1\text{-b}}$ (Mr 8 kDa) were isolated from P_1 by gel filtration on Sephadex G-200. They inhibited the activity of HIV-1 RT with an IC50 of 158 nM and 148.16 μg mL^{-1} (18.5 μM), respectively. Further structural analyses revealed that $P_{1\text{-a}}$ was a polysaccharide–peptide complex, and $P_{1\text{-b}}$ was a polymer consisting of acteoside and acteoside derivatives identified by Fourier transform infrared spectroscopy, nuclear magnetic resonance, assays of carbohydrate and protein contents and high-performance liquid chromatography electrospray ionization mass spectrometry.

Introduction

Acquired immune deficiency syndrome (AIDS), caused by the human immunodeficiency virus type 1 (HIV-1) retrovirus (Barre-Sinoussi et al 1983; Popovic et al 1983), has become an epidemic of global proportions, with an estimated 35 to 42 million people surviving with the disease at the end of 2004 (Piot et al 2001; World Health Organization 2005). Three enzymes are essential to the life cycle of HIV-1, namely HIV-1 reverse transcriptase (RT), HIV-1 protease and HIV-1 integrase. Chemotherapeutic strategies have been focused on the development of inhibitors against these viral enzymes. HIV-1 RT is an attractive target in the treatment of AIDS, for which no completely successful chemotherapy is yet available (Jacobo-Molina & Arnold 1991; Yeni et al 2002; De Clercq 2004).

Traditional Chinese medicinal herbs have been shown to be effective in inhibiting the growth of HIV in-vitro (Chang & Yeung 1988; Lu 1995; Wang & Ng 2000, 2001, 2004). Further analysis of these natural products in various enzyme assays demonstrated that some extracts from Chinese herbs exerted their effects at different stages in HIV-1 replication. For example, Collins et al (1997) reported that 6 out of the 19 aqueous herbal extracts caused significant inhibition of the interaction between HIV-1 gp120 and immobilized CD4 receptors. Several extracts were also capable of inhibiting the activity of recombinant HIV-1 RT. It is therefore worthwhile to search for natural products with anti-HIV-1 RT activity. The objective of the present investigation was to screen 18 medicinal herbal extracts for in-vitro inhibition of recombinant HIV-1 RT and to purify and chemically characterize the active principles $P_{1\text{-a}}$ and $P_{1\text{-b}}$ from *Rosa rugosa*.

Rugosa rose (*R. rugosa Thunb.*) and its horticultural variety (*R. rugosa* var. *plena*) are reputed as aromatic and medicinal plants in China. The traditional use of rose flowers in China is for relieving pain and diarrhoea, and for the treatment of neurotic gastritis, chronic gastritis, hepatitis, acute mastitis, trauma and blood disorders (Hsu et al 1986); it is also used as an antidiarrhoeal and haemostatic agent in northern Japan (Hashidoko 1996). *R. rugosa* is a rich source of phytochemicals. Flavonoids isolated from the rose display antioxidative

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Funding: We thank the National Natural Science Foundation (grant no. 30570051) and Tianjin Natural Science Foundation (grant no. 06YFJMJCO7500) for financial support.

activity (Kleszczynska et al 1999). The secondary metabolites of *R. rugosa*, defined by structural class, comprise hydrolysable tannins (contained in foliage and petals), flavonoids (in leaves), 2-phenoxychromones (in foliage), catechin derivatives (roots), monoterpenes (in floral parts, foliage), sesquiterpenes (in leaves, especially from glandular trichomes) and triterpenes (in foliage and roots) (Hashidoko 1996). Secondary metabolites of plants play a role as chemical messengers or as anti-pest agents (Harborne 1993). From dried rose flowers, a gallic acid derivative (Ng et al 2004) and a polysaccharopeptide (Ng et al 2006) with antioxidant activity have been isolated. A fraction that appears to be a condensed tannin has also been isolated from the ethanol precipitate of a hot water extract of rose flowers. The fraction, designated as $P_{1\text{-}b}$, was adsorbed on DEAE-cellulose and eluted by 0.5 M NaCl, and subsequently eluted from the Sephadex G200 column as the major peak. Fraction $P_{1\text{-}b}$ exerted a dose-dependent inhibitory action on lysis of mouse erythrocytes (Sugiyama et al 1993) (almost complete inhibition, 76% inhibition and 50% inhibition at 250, 125 and 62.5 μg mL^{-1}, respectively). It suppressed lipid peroxidation in mouse brain and kidney homogenates (Liu et al 1997) (27% and 12% inhibition at 250 μg mL^{-1}, respectively). It also increased the activity of superoxide dismutase in erythrocytes (Giannopolitis & Ries 1997) from 1.24 units to 1.52 units at 500 μg mL^{-1} (Ng et al 2006). The aim of the present study was to chemically characterize fraction $P_{1\text{-}b}$. The HIV-1 RT inhibitory activity of fraction $P_{1\text{-}b}$ and of the polysaccharopeptide fraction $P_{1\text{-}a}$ is reported here.

Table 1 Extracts and ethanol precipitates of Chinese medicinal herbs for inhibition of human immunodeficiency virus type 1 reverse transcriptase activity

Plant species	% Inhibition at 500 μg mL^{-1}	
	Aqueous extract	Ethanol precipitate
Eucommia ulmoides	62.0 ± 0.6	54.0 ± 2.7
Paeonia lactiflora	76.5 ± 2.3	48.5 ± 5.1*
Angelica sinensis	27.1 ± 4.2	35.1 ± 1.9
Atractylodes macrocephala	5.9 ± 0.8	13.9 ± 2.4*
Astragalus membranaceus	9.5 ± 4.8	4.3 ± 0.8
Polygonatum odoratum	13.7 ± 2.8	11.9 ± 2.1
Mornida officinallis	−10.1 ± 3.5	−5.1 ± 2.6
Chrysanthemum morifolium	62.8 ± 5.3	63.6 ± 6.1
Ligusticum wallichil	51.4 ± 2.3	61.5 ± 2.4*
Epimedium sagitlatum	81.8 ± 7.3	75.3 ± 2.7
Stachys sieboldii	−0.1 ± 0.5	2.6 ± 0.1
Salvia miltiorrhiza	81.1 ± 5.4	75.7 ± 4.7
Lycium chinense	−2.2 ± 0.3	4.5 ± 0.1
Rosa rugosa	83.2 ± 2.1	87.9 ± 3.4
Achyranthes bidentata	27.4 ± 3.5	32.4 ± 2.9
Ficus carica	−12.8 ± 2.8	12.8 ± 2.3*
Atractylodes lancea	17.9 ± 4.8	21.6 ± 1.6
Tribulus terrestris	73.7 ± 5.9	54.6 ± 4.1*

Data are mean ± s.e.m., n = 3. *$P < 0.05$, significantly different compared with the corresponding aqueous extract (Student's *t*-test).

Materials and Methods

Preparation of samples

The 18 dried medicinal herbs analysed in this study (see Table 1) were purchased from a local herbal shop. The herbs were identified by a herbal specialist at the shop and vouchers are kept in the Department of Microbiology, Nankai University, Tianjin, China. Each of the dried samples (2 g) was extracted with 10 mL boiling distilled water under reflux for 2 h. After filtration to remove insoluble debris, the water extract was lyophilized. Dried water extracts were dissolved in distilled water, mixed with 4 vols of ethanol, and then kept overnight in a refrigerator at 4°C. The precipitate was collected by centrifugation and then lyophilized.

Assay for HIV-1 RT inhibitory activity

The assay for HIV-1 RT inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer-Mannheim (Germany). The assay takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly(A) oligo(dT)$_{15}$. The digoxigenin- and biotin-labelled nucleotides in an optimized ratio are incorporated into the same DNA molecule, which was freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity followed a sandwich enzyme-linked immunosorbent assay (ELISA) protocol. Biotin-labelled DNA binds to the surface of microtitre plate modules that have been pre-coated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labelled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyses the cleavage of the substrate, producing a coloured reaction product. The absorbance of the samples at 405 nm can be determined by using a microtitre plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the test sample was calculated as percentage inhibition compared with a control without the sample (Collins et al 1997; Wang & Ng 2001). *Phaseolus vulgaris* bean phytohemagglutinin Wang & Ng (2001) was used as a positive control in the assay.

Purification by anion exchange chromatography on DEAE-cellulose

The ethanol precipitate of the aqueous extract of dried rose (*R. rugosa Thunb.*) flowers (50 mg) was dissolved in 10 mM Tris-HCl buffer (pH 6.5) before centrifugation to remove insoluble material. The solution was chromatographed on a 2.5 × 35-cm column of DEAE-cellulose (Sigma) equilibrated and eluted with 10 mM Tris-HCl buffer (pH 6.5). After eluting unadsorbed material with the same buffer, the column was eluted with a linear gradient of 0 to 2 M NaCl solution in the buffer. Absorbance at 280 nm was measured. Two fractions (P_1 and P_2) were collected and dialysed against deionized water before lyophilization.

Further purification by column chromatography on Sephadex G-200 and molecular weight determination

The P_1 powder was applied to a 2.5×100-cm column of Sephadex G-200 (Amersham Biosciences) (fractionation range 5–600 kDa) in distilled water and was eluted with distilled water. The elute was monitored with a UV detector at 280 nm. The molecular weight of the fraction was determined by gel filtration on Sephadex G-200. Bovine serum albumin, egg albumin, and cytochrome C were used as standards and distilled water was used as eluent. The elution volume of dextran blue 2000 was used as void volume.

Chemical analysis

The carbohydrate and protein contents of the fraction were quantitatively determined by the colorimetric anthrone-H_2SO_4 method (Kawagishi et al 1990) and Folin phenol method (Lowry et al 1951), respectively.

High-performance liquid chromatography (HPLC)

After the two aforementioned chromatographic steps, the active fractions were subjected to reverse-phase HPLC, which was conducted using an ODS C18 column (19×300 mm) and an LC-A4 HPLC. The buffer used was 60% methanol/40% Na_2HPO_4-KH_2PO_4 (0.025 M, pH 6.5). The flow rate was 30 mL h^{-1}.

Fourier transform infrared (FTIR) spectroscopy

A 2-mg sample was ground in mortar. KBr powder (200 mg), which had been ground to approximately 2 μm and dried in an oven at 110–150°C for 48 h, was then added and ground evenly together with the sample. The mixture was compressed in vacuum to form a semi-transparent tablet with a diameter of 5 mm or 13 mm. The tablet was placed in a sample window of an FTIR spectrometer (Magna-560). A KBr tablet was placed in the reference window. Spectroscopy was then performed.

Nuclear magnetic resonance (NMR) spectroscopy

A 10-mg sample was dissolved in 250–300 μL D_2O. After filtration, the solution was transferred to a sample tube (5 mm diam.), and placed in a sample window of a Mercury-300BB NMR spectrometer. Spectroscopic measurements were then performed. Five-millimetre gradient reverse $^1H/^{13}C$ double probes were used. The working frequencies for ^1H and ^{13}C were 300.07 MHz and 75.45 MHz, respectively.

HPLC-electrospray ionization mass spectrometry (ESI-MS)

To prepare the sample, 1 mg $P_{1\text{-}b}$ and 0.6 mL of water were added to 6 mL of a freshly prepared mixture of 1-butanol: hydrochloric acid (95:5, v/v) in a 10-mL screw-top glass centrifuge tube. The optimal concentration of water in the reaction mixture was 10–11% (v/v). After mixing, tubes were placed in a water bath at 95°C for 50 min. Tubes were then cooled to room temperature.

Fragment identification after hydrolysis of $P_{1\text{-}b}$ was performed by HPLC-ESI-MS analysis. The HPLC system consisted of a Waters (Milford, MA, USA) 2690 HPLC system with a photodiode-array detector set at 265 nm. The chromatographic conditions were as follows: column, ODS C_{18}, 250 mm× 4.6 mm, 5 μm; eluent: (A) methanol and (B) 0.5% acetic acid. The linear gradient was 5–70% A for 40 min, 70–100% A for 10 min and 100% A for 10 min; the flow rate was 0.6 mL min^{-1}; and the temperature was 25°C.

The mass spectrometry determination was performed on an LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) with an electrospray source in the positive ion mode. The electrospray voltage was set to 4.0 kV. The capillary temperature was 250°C. The HPLC system was connected to the mass spectrometer via the UV cell outlet.

Results

Inhibition of HIV-1 RT activity

The HIV-1 RT inhibitory activity of the aqueous extracts and the subsequent ethanol precipitates of 18 medicinal herbs are presented in Table 1. The screening test was carried out at a sample concentration of 500 μg mL^{-1}. The results showed that the ethanol precipitate of the aqueous extract of dried rose flowers had the highest inhibitory activity against HIV-1 RT.

Purification and molecular weight determination of fractions from *R. rugosa* flowers

P was chromatographed on DEAE-cellulose. The components adsorbed on DEAE-cellulose were eluted with a linear gradient of 0 to 2 M NaCl solution. Two components, designated as P_1 and P_2, were eluted at a concentration of 0.5 M NaCl and 1 M NaCl solution, respectively. The carbohydrate content of P_1 was higher than that of P_2, as indicated by OD 620 (data not shown).

Gel filtration of P_1 on Sephadex G-200 yielded two fractions, $P_{1\text{-}a}$ and $P_{1\text{-}b}$. $P_{1\text{-}a}$ was a white powder and gave a very broad, small single peak on Sephadex G-200, with a molecular weight of about 150 kDa. $P_{1\text{-}b}$ was a filemot powder and gave a single, sharp peak on Sephadex G-200 with a molecular weight of about 8 kDa.

Inhibition of HIV-1 RT activity by fractions from *R. rugosa*

P, P_1, $P_{1\text{-}a}$ and $P_{1\text{-}b}$ produced different extents of inhibition of HIV-1 RT at a concentration of 100 μg mL^{-1}. $P_{1\text{-}a}$ showed the strongest inhibition. The IC50 values of $P_{1\text{-}a}$ and $P_{1\text{-}b}$ were 23.72 μg mL^{-1} (158 nM) and 148.16 μg mL^{-1} (18.5 μM), respectively (Figure 1).

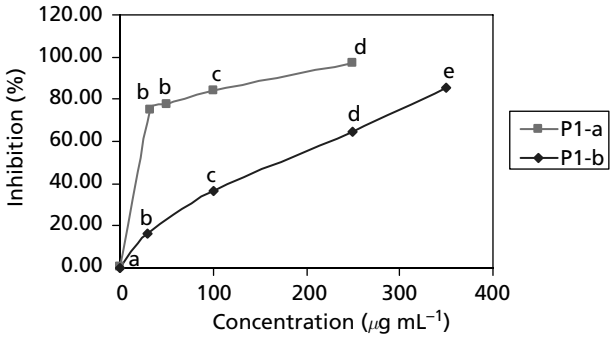

Figure 1 Concentration-dependent inhibition of HIV-1 RT by $P_{1\text{-}a}$ and $P_{1\text{-}b}$. Each point represents the mean of three separate measurements. The s.e.m. of each data point was less than 3% of the mean. Different letters indicate statistically significant differences ($P < 0.05$; analysis of variance followed by Duncan's multiple range test). IC50 = 23.72 μg mL^{-1} or 158 nM. By comparison, P and P_1 produced 67.84 ± 1.13% and 82.73 ± 0.87% inhibition (mean ± s.e.m., n = 3), respectively, when tested at 100 μg mL^{-1}.

Chemical characterization of active compounds

The carbohydrate/protein ratio for $P_{1\text{-}a}$ was 8.6:1, according to results of structural analysis by FTIR, NMR and HPLC-ESI-MS. It could be deduced that $P_{1\text{-}a}$ was a polysaccharide–peptide complex of the α-type bond type and $P_{1\text{-}b}$ was a polymer consisting of acteoside and acteoside derivatives.

$P_{1\text{-}a}$ yielded a peak with a small hillock when subjected to HPLC. The results showed that $P_{1\text{-}a}$ was a polysaccharide–peptide complex. $P_{1\text{-}b}$ yielded a single peak when subjected to HPLC. The FTIR spectrum of $P_{1\text{-}a}$ showed that 3422 cm^{-1} ($\nu_{O\text{-}H}$ and $\nu_{N\text{-}H}$) and 2932 cm^{-1} ($\nu_{C\text{-}H}$) were the regions of characteristic absorbance of the polysaccharide. In addition, 1654–1624 cm^{-1} ($\nu_{C=O}$), 1404 cm^{-1} ($\nu_{C\text{-}O}$) and 1019 cm^{-1} ($\nu_{C\text{-}O}$) were also the regions of absorbance of carbohydrates. Absorbance at 833.9 cm^{-1} ($\nu_{C\text{-}H}$) indicated that the bond type was α-type (data not shown).

The FTIR spectrum of $P_{1\text{-}b}$ showed that 3423 cm^{-1} ($\nu_{O\text{-}H}$) and 2932 cm^{-1} ($\nu_{C\text{-}H}$) were the characteristic regions of absorbance of carbohydrates. The results were consistent with the assay of total contents of carbohydrate and protein. In addition, the absorbance of 1743 cm^{-1}, 164 cm^{-1} and 1300–1000 cm^{-1} indicated the presence of C=O, C-C and C-O in $P_{1\text{-}b}$, respectively (data not shown).

In the ^{1}H NMR spectrum of $P_{1\text{-}b}$, many peaks appeared at δ5.0 ppm and δ3.5–4.5 ppm, indicating the presence of proton of glucose ring. The peak at δ175.58 ppm in the ^{13}C-NMR spectrum of $P_{1\text{-}b}$ indicated the presence of C=O. The peaks at δ55–80 ppm showed the presence of C-O, C-N or C-H (data not shown).

The chemical analysis, NMR spectrum and FTIR spectrum showed that $P_{1\text{-}b}$ was a complex fraction with high molecular weight; it was difficult to infer the structure. HPLC-ESI-MS/MS was used to analyse the structure of fragments produced by the hydrolysis of $P_{1\text{-}b}$.

The HPLC-MS of peak 1 (t_R = 1.19 min) yielded m/z 477 as the deprotonated molecular ion [M-H]$^-$. Further experiments in the MS/MS of the m/z 477 ([M-H]$^-$) produced two main fragment ions at m/z 315 and 461 (data not shown). The compound corresponding to peak 1 was therefore identified as demethyl-glucoacteoside by comparing its congruent mass spectral data with that of the standard (Table 2).

The HPLC-MS of peak 2 (t_R = 6.12 min) gave m/z 623 as the deprotonated molecular ion [M-H]$^-$. Further experiments in the MS/MS of the m/z 623 ([M-H]$^-$) produced main fragment ions at m/z 461 (data not shown). The compound corresponding to peak 2 was identified as acteoside by comparing its congruent mass spectral data with that of the standard (Table 2).

The HPLC-MS of peak 3 (t_R = 9.86 min) gave m/z 665 as the deprotonated molecular ion [M-H]$^-$. Further experiments in the MS/MS of the m/z 665 ([M-H]$^-$) produced fragment ions at m/z 315, 461 and 161 (data not shown). The compound corresponding to peak 3 was identified as 2′-acetylacteoside by comparing its congruent mass spectral data with that of the standard (Table 2).

The HPLC-MS of peak 4 (t_R = 23.74 min) gave m/z 785 as the deprotonated molecular ion [M-H]$^-$. Further experiments on the MS/MS of the m/z 785 ([M-H]$^-$) produced one main fragment ion at m/z 623 (data not shown). The compound corresponding to peak 4 was identified as echinacoside by comparing its congruent mass spectral data with that of the standard (Table 2).

The above results indicate that $P_{1\text{-}b}$ is a polymer consisting of acteoside and acteoside derivatives.

Discussion

Natural products from traditional medicinal herbs have been reported to have inhibitory effects on HIV-1 RT (Collins et al 1997; Matsuse et al 1999; Wang & Ng 2004). In the present study, we examined the anti-HIV-1 RT activity of 18 medicinal herbs. The herbal extracts showed differential inhibition of HIV-1 RT activity.

Compounds isolated from the ethanol precipitate of an aqueous extract of *R. rugosa* flowers showed anti-HIV-1 RT activity. The inhibitory effect of *Rosa* sp. against HIV has been previously demonstrated: Mahmood et al (1996) found that the water and methanol extracts of *Rosa damascena* exhibited moderate anti-HIV activity. They also compared the anti-viral

Table 2 Fragment ions observed (m/z) in the electrospray ionization mass spectra from standards by using [M-H]$^-$ as the precursor ions.

Compound	Precursor ion [M-H]$^-$ (m/z)			Product ion (m/z)					
Acteoside	623			461	315	179	161	153	135
2′-Acetylacteoside	665	623		461	315	179	161	153	135
Demethyl-glucoacteoside	477			461	315	179	161	153	135
Echinacoside	785	623	477	461	315	179	161	153	135

activities of nine compounds isolated from its methanol extracts, which were totally different in structure from $P_{1\text{-}a}$ and $P_{1\text{-}b}$.

NMR and FTIR spectra showed that $P_{1\text{-}b}$ is a complex component and it is difficult to infer the structure from NMR and FTIR spectroscopy. Therefore, HPLC-ESI-MS was used to analyse $P_{1\text{-}b}$. Structural assays (FTIR, NMR and HPLC-ESI-MS) indicated that $P_{1\text{-}b}$ was a polymer consisting of acteoside and acteoside derivatives.

Acteoside and acteoside derivatives belonging to phenylethanoid glycosides are phenylpropanoid derivatives. Phenylethanoid glycosides have strong antioxidant and free radical scavenging activities (Wang et al 2000). A number of phenylpropanoid derivatives, such as eugenol and 4-methyleugenol, have been isolated from the petals and pollen of *R. rugosa* (Hashidoko 1996). However, the phenylpropanoid derivatives found were all of small molecular weight. In this study, $P_{1\text{-}b}$ was found to be a polymer consisting of acteoside and acteoside derivatives with a molecular weight of 8 kDa.

$P_{1\text{-}a}$ is a polysaccharide–peptide complex. Previous studies have focused on the immunomodulatory and anti-tumour activities of polysaccharide–peptides (Liu et al 1998; Tsang et al 2003). There are very few reports on the inhibitory effects of polysaccharide–peptides on HIV-1 RT. $P_{1\text{-}b}$ is a polymer comprised of acteoside and acteoside derivatives. It has been reported that acteoside shows anti-hepatotoxic (Xiong et al 1998), anti-inflammatory, antinociceptive (Schapoval et al 1998), and antioxidant (Wong et al 2001) activities. This is the first report that acteoside has an inhibitory effect on HIV-1 RT. The strong inhibitory effect of $P_{1\text{-}a}$ on HIV-RT, coupled with its high solubility in water, heat stability and low cytotoxicity, make it a useful compound for further studies on its possible use as an anti-viral agent in-vivo. Although $P_{1\text{-}b}$ elicits moderate inhibition of HIV-1 RT, it also exhibits antioxidant activity. In fact, nucleoside analogue RT inhibitors have severe side-effects, especially oxidative stress (Cote et al 2002; De la Asuncion et al 2004). Thus, medicinal herbs constitute a large and promising source of anti-HIV compounds. Whether these compounds have less untoward side-effects awaits further study.

In conclusion, in this study, a polysaccharide–peptide complex and a polymer consisting of acteoside and acteoside derivatives were isolated from dried rose flowers. Both inhibited the activity of HIV-1 RT.

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