Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase and Ribonuclease H Activities by Constituents of *Juglans mandshurica*

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From the stem-bark of *Juglans mandshurica*, two new naphthalenyl glucopyranosides, 1,4,8-trihydroxynaphthalene 1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside] (1) and 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,5″-dihydroxy-4″-methoxybenzoyl)]glucopyranoside (4), and two new α-tetralonyl glucopyranosides, 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,5″-dihydroxy-4″-methoxybenzoyl)]glucopyranoside (7) and 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,4″,5″-trihydroxybenzoyl)]glucopyranoside (8), were isolated together with three known naphthalenyl glucopyranosides (2, 3 and 5), one α-tetralonyl glucopyranoside (6), four flavonoids (9—12), and two galloyl glucopyranosides (13, 14).

Amongst the isolated compounds, 1,2,6-trigalloylglucopyranose (13) and 1,2,3,6-tertagalloylglucopyranose (14) exhibited the most potent inhibition of reverse transcriptase (RT) activity with IC_{50} values of 0.067 and 0.040 μM, respectively, while the latter compound also inhibited ribonuclease H (RNase H) activity with an IC_{50} of 39 μM, comparable in potency to illimaquinone used as a positive control. 1,4,8-Trihydroxy-naphthalene 1-*O*-β-D-glucopyranoside (2), 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (3) and 8 showed moderate inhibition against both enzyme activities, and inhibitory potency of 2 against RNase H activity (IC_{50}=156 μM) was slightly greater than that against the RT activity (IC_{50}=290 μM). The inhibitory potencies of 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethoxybenzoyl)] glucopyranoside (6), 7 and 8 against RT activity increased accompanied by an increase in the number of free hydroxyls on the galloyl residues, as represented by the IC_{50} values of >500, 330 and 5.8 μM, respectively.

Key words human immunodeficiency virus; *Juglans mandshurica*; naphthalenyl glucopyranoside; reverse transcriptase; ribonuclease H; α-tetralonyl glucopyranoside

Reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) has been demonstrated to be important for viral replication.[1,2] The crucial role of RT in the early stages of the HIV-1 life cycle has made it one of the most reliable targets for potential anti-AIDS chemotherapy.[3] The enzyme possesses not only RT activity but also DNA-dependent DNA polymerase and ribonuclease H (RNase H) activities. The single-stranded RNA genome of HIV is reverse-transcribed by the RNA-dependent DNA polymerase activity (RT activity) into the minus DNA strand to form an RNA–DNA hybrid. Then, RNase H catalyzes hydrolysis of the RNA component of the hybrid leaving small RNA primers for the subsequent synthesis of complementary plus DNA strand by the DNA-dependent DNA polymerase activity.[4,5] The enzyme from HIV-1 consists of a pair of polypeptides, in which the DNA polymerase activity resides in the *N*-terminal domain, whereas the RNase H activity is located in the *C*-terminal domain.[6]

Inhibition of each catalytic function of RT interferes with virus production. Two classes of inhibitors of RT have been developed up to the present; nucleoside analogues and non-nucleoside compounds, which are distinguished by their different inhibitory mechanisms. The nucleoside analogues 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dideoxycytidine (DDC) and 2′,3′-dideoxyinosine (DDI) act by chain termination and are known to inhibit competitively with respect to substrates, deoxynucleoside triphosphates. Unfortunately, their use for treatment of patients with AIDS is limited due to emergence of resistant virus and their cellular toxicity. On the other hand, non-nucleoside inhibitors act at sites other than the substrate binding sites of the polymerase.[7] These include compounds, such as nevirapine,[7] calanoide,[8] coumarin derivatives,[9] benzodiazepine derivatives,[10] pyridinone,[11] catechin derivatives[12] and psychotrine.[13] These inhibitors have been reported to exert low levels of toxicity. However, these compounds also lead to rapid drug cross-resistance. The need for development of an effective and selective inhibitor of HIV-1 with a new mechanism of action still remains. A relatively large volume of research has been conducted on the inhibition of RT activity, but there are only a few reports on the selective inhibition of RNase H activity, such as herparin,[14] illimaquinone,[15] novenamines (U-34445, U-35122 and U-35401)[16] and a degradation product of cephalosporin (HP 0.35).[17]

Therefore, we examined a conventional assay method for RNase H activity associated with HIV-1 RT to find new inhibitory substances from natural sources. During *in vitro* screening, we found that naphthoquinone derivatives and naphthalene derivatives from *Lithospermum erythrorhizon* Siebold *et* Zuccarini (Borraginaceae), *Limonium tetragonum* A. A. Bullock (Plumbaginaceae), and *Juglans* (*J.*) *mandshurica* Maximowicz (Juglandaceae) inhibited RNase H activity. Of the tested plants, the extract of *J. mandshurica* (stem-bark) appreciably inhibited RNase H activity with a 50% inhibitory concentration (IC_{50}) of 22 μg/ml, while it more potently inhibited RT activity (IC_{50}, 0.047 μg/ml).

J. mandshurica has been used as a folk medicine for treatment of cancer in Korea.[18] Several naphthoquinones, naph-

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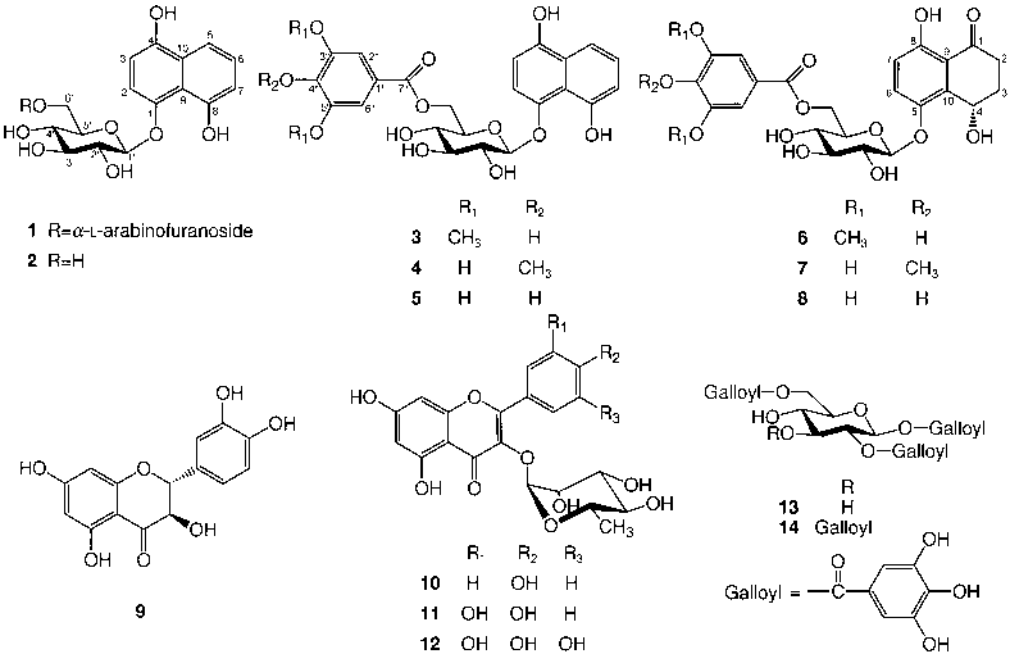

Chart 1. Structures of Isolated Compounds

thalenyl glucopyranosides, α-tetralonyl glucopyranosides and diarylheptanoyl glucopyranosides have been isolated from this plant[18—20] and these compounds have been shown to display cytotoxicity to human colon and lung carcinoma.[20] In this paper, we describe the characterization of compounds isolated from the stem-bark of this plant and their inhibitory potencies against HIV-1 RT and RNase H activities.

Results and Discussion

Isolation and Structure Determination Repeated column chromatography of an EtOAc-soluble fraction of the MeOH extract of *J. mandshurica* (stem-bark) on silica gel followed by gel filtration on Sephadex LH-20 and reversed phase medium pressure liquid chromatography (MPLC) led to the isolation of five naphthalenyl glucopyranosides (**1**—**5**), three α-tetralonyl glucopyranosides (**6**—**8**), four flavonoids (**9**—**12**) and two galloyl glucopyranosides (**13**—**14**). The known compounds were identified as 1,4,8-trihydroxynaphthalene 1-*O*-β-D-glucopyranoside (**2**),[21] 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (**3**),[19] 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,4″,5″-trihydroxybenzoyl)]glucopyranoside (**5**),[19] 4,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(4″-hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (**6**),[20] taxifolin (**9**),[22] afzelin (**10**),[23] quercitrin (**11**),[24] myricitrin (**12**),[24] 1,2,6-trigalloylglucopyranose (**13**)[25] and 1,2,3,6-tertagalloylglucopyranose (**14**)[26] by comparing their spectral data with those previously reported.

Compound **1** was obtained as a light brown amorphous powder, $[\alpha]_D$ −94°. Its positive FAB-MS spectrum gave a quasi-molecular ion peak at *m/z* 471 $[M+H]^+$. The ^{1}H-NMR spectrum showed signals for five aromatic protons at δ 6.70, 6.81, 7.21, 7.26 and 7.65, eleven sugar protons between δ 3.42 to 4.10, and two anomeric protons at δ 4.95 and 4.97 (Table 1). The ^{13}C-NMR spectrum, in combination with distortionless enhancement by polarization transfer (DEPT) and ^{1}H-detected multiple quantum coherence (HMQC) experiments, showed signals for five aromatic methine carbons at δ 108.3, 112.1, 113.0, 114.6 and 127.1, seven aliphatic methine carbons in a region at δ 71.8 to 85.6, two anomeric carbons at δ 105.2 and 110.0, two aliphatic methylenes at δ 62.9 and 68.3, and five quaternary carbons (Table 2). On the basis of spectroscopic evidence obtained by ^{1}H–^{1}H correlation spectroscopy (COSY) and HMQC experiments, all protons and carbons of sugars were assigned as shown in Tables 1 and 2,

Table 1. ^{1}H-NMR Spectral Data of Compounds **1** and **4** in CD_3OD

Position	**1**[a]	**4**[b]
1		
2	6.70 d (8.4)	6.44 d (8.2)
3	7.21 d (8.4)	7.06 d (8.2)
4		
5	7.65 dd (8.4, 1.1)	7.55 dd (8.3, 1.1)
6	7.26 dd (8.4, 7.5)	7.16 dd (8.3, 7.6)
7	6.81 dd (7.5, 1.1)	6.71 dd (7.6, 1.1)
8		
9		
10		
1′	4.95 d (7.7)	4.87 d (7.5)
2′	3.53 m	3.49 m
3′	3.47 m	3.43 m
4′	3.42 dd (17.8, 8.6)	3.41 m
5′	3.64 m	3.71 m
6′	3.68 m	4.42 dd (11.8, 7.0)
	4.10 m	4.58 dd (11.8, 2.2)
1″	4.97 d (1.3)	
2″	4.04 dd (3.4, 1.3)	7.05 s
3″	3.86 dd (5.9, 3.4)	
4″	3.99 m	
5″	3.63 dd (11.8, 5.1)	
	3.73 dd (11.8, 3.2)	
6″		7.05 s
7″		
3″,5″-OCH_3		
4″-OCH_3		3.82 s

a) 500 MHz. *b*) 400 MHz.
δ values in ppm and coupling constants (in parentheses) in Hz.

Table 2. ^{13}C-NMR Spectral Data of Compounds **1**, **4** and **6**—**8** in CD_3OD

Position	**1**[a]	**4**[b]	**6**[a]	**7**[b]	**8**[b]
1	148.5	148.6	206.3	206.4	206.4
2	108.3	108.4	33.5	33.5	33.5
3	113.0	113.3	30.2	30.2	30.2
4	150.5	150.5	61.3	61.3	61.3
5	114.6	114.6	148.3	148.5	148.5
6	127.1	127.1	128.7	128.8	128.8
7	112.1	112.2	118.8	119.1	119.2
8	154.7	154.6	159.2	159.3	159.3
9	117.8	117.8	116.1	116.4	116.1
10	128.8	128.9	135.4	135.2	135.1
1′	105.2	105.3	104.2	104.6	104.6
2′	75.1	75.1	75.2	75.3	75.3
3′	78.1	78.1	77.8	78.0	77.9
4′	71.8	71.8	71.9	71.9	71.7
5′	77.2	76.0	75.8	75.7	75.8
6′	68.3	65.1	65.0	64.9	64.6
1″	110.0	126.5	121.1	126.4	121.0
2″	83.4	110.4	108.3	110.2	110.1
3″	78.8	151.8	148.9	151.9	146.7
4″	85.6	141.4	142.3	141.5	140.4
5″	62.9	151.8	148.9	151.9	146.7
6″		110.4	108.3	110.2	110.1
7″		167.7	167.7	167.7	168.2
3″,5″-OCH_3			56.9		
4″-OCH_3	148.5	60.8		60.8	

a) 125 MHz. *b*) 100 MHz.

respectively.[27,28]

The sugar linkages were determined by heteronuclear multiple-bond correlations (HMBC, Fig. 1), which showed correlations between signals at δ_H 4.95 (Glc-H-1′) and at δ_C 148.5 (C-1 of the aglycone), and at δ_H 4.97 (Ara-H-1″) and δ_C 68.3 (Glc-C-6′), indicating glycosylation at C-1 with an Ara (1→6)Glc moiety. The positions of the hydroxy groups on the naphthalene ring were deduced from the coupling pattern in the ^{1}H-NMR spectrum and the correlations between signals at δ_H 6.70 (H-2) and 7.21 (H-3), and among signals at δ_H 7.65 (H-5), 7.26 (H-6) and 6.81 (H-7) in the ^{1}H–^{1}H COSY spectrum. This was further confirmed by an HMBC experiment, where long-range correlations were observed between signals at δ_H 6.70 (or 7.21) and δ_C 150.5 (C-4), and at δ_H 7.26 (or 6.81) and δ_C 154.7 (C-8) (Fig. 1).

Acid hydrolysis of **1** afforded glucose and arabinose as monosaccharide units, which were identified on TLC by comparison with authentic samples. Furthermore, these sugars were determined to be D-glucose and L-arabinose, respectively, by GLC of their pertrimethylsilated L-cysteine methyl ester derivatives.[29] The configuration of the glycosidic linkage of the glucopyranose moiety in **1** was determined to be β on the basis of the $J_{1',2'}$ value (7.7 Hz) of the anomeric proton, while that of the arabinofuranose moiety was α from the $J_{1'',2''}$ value (1.3 Hz) and the chemical shifts of C-1 (δ_C 110.0) and C-2 (δ_C 83.4) in the ^{13}C-NMR spectrum.[27,28] Consequently, the structure of **1** was determined as 1,4,8-trihydroxynaphthalene 1-*O*-[α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside].

Compound **4**, a light brown amorphous powder, gave a quasi-molecular ion peak $[M+H]^+$ at *m/z* 505 in the positive FAB-MS spectrum. The ^{1}H- and ^{13}C-NMR spectra of **4** were similar to those of **1**, except for signals due to a methylated galloly moiety instead of the arabinose signals. It showed a

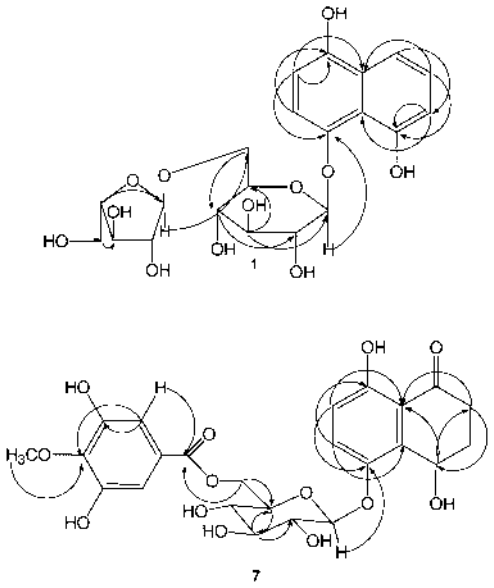

Fig. 1. HMBC Correlation of **1** and **7**

methoxy proton at δ 3.82 and an aromatic proton at δ 7.05 in the ^{1}H-NMR spectrum (Table 1). The methylated galloyl moiety was deduced from the chemical shift of the methoxy carbon at δ 60.8, two chemically equivalent aromatic carbons at δ 110.4 (C-2″, 6″) and 151.8 (C-3″, 5″), two substituted aromatic carbons, and one carbonyl carbon at δ 167.7 in the ^{13}C-NMR spectrum (Table 2). The chemical shift values and coupling constants of the glucosylnaphthalene moiety were essentially identical to those of **1**. The glycone part was identified as glucose on TLC after acid hydrolysis. The position linked with the galloyl group was determined by HMBC, which showed correlations between signals at δ_H 4.42 (H-6′) and δ_C 167.7 (C-7″), as well as between signals at 7.05 (H-2″, 6″) and δ_C 167.7 (C-7″). The position of a methoxy group on the galloyl moiety was assigned at C-4″ by observation of an equivalent signal in the ^{1}H-NMR spectra (δ 7.05; H-2″, 6″) and the ^{13}C-NMR (δ 110.4; C-2″,6″ and 151.8; C-3″,5″). Furthermore, it was confirmed by the presence of correlations of ^{1}H-signals at δ_H 7.05 (H-2″,6″) and 3.82 (OCH_3) with a ^{13}C-signal at δ_C 141.4 (C-4″) in the HMBC spectrum. The configuration of the glucosidic linkage was assigned as β on the basis of the coupling constant, which was similar to that in **1**. The structure of **4** was thus determined to be 1,4,8-trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,5″-dihydroxy-4″-methoxybenzoyl)]glucopyranoside.

Compound **7**, a light yellow amorphous powder, showed a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 545 in the positive FAB-MS spectrum. The ^{1}H- and ^{13}C-NMR spectra of **7** were similar to those of **4**. However, it had signals assignable to two methylenes at δ 2.16 (2H), 2.48 (1H) and 3.01 (1H), and an oxymethylene at δ 5.32 (t, J=3.3 Hz) in the ^{1}H-NMR spectrum (Table 3), and signals for a carbonyl carbon at δ 206.4, two methylene carbons at δ 30.2 and 33.5, and a carbinol carbon at δ 61.3 in the ^{13}C-NMR spectrum (Table 2), indicating an oxygenated α-tetralone moiety in **7**. The presence of this aglycone was further confirmed by the positive FAB-MS spectrum, which showed a prominent fragment ion peak at *m/z* 329 $[M-$tetralone moiety$]^+$.[20] The sugar linkages and the methylated galloyl group were determined

Table 3. ^{1}H-NMR Spectral Data of Compounds **6**—**8** in CD_3OD

Position	**6**[a]	**7**[b]	**8**[b]
1			
2β	2.44 dt (17.7, 3.4)	2.48 dt (17.7, 3.4)	2.48 dd (17.6, 3.4)
2α	3.00 ddd (17.7, 12.6, 5.6)	3.01 ddd (17.7, 11.8, 6.8)	3.01 ddd (17.6, 11.9, 6.5)
3	2.13 m	2.16 m	2.16 m
4	5.30 t (3.2)	5.32 t (3.3)	5.32 t (3.2)
5			
6	7.36 d (9.1)	7.40 d (9.2)	7.41 d (9.2)
7	6.60 d (9.1)	6.74 d (9.2)	6.75 d (9.2)
8			
9			
10			
1′	4.81 d (7.5)	4.78 d (7.7)	4.78 d (7.7)
2′	3.54 m	3.54 m	3.55 t (8.2)
3′	3.50 m	3.48 m	3.49 m
4′	3.43 m	3.44 m	3.45 m
5′	3.70 ddd (9.5, 7.3, 2.2)	3.67 ddd (9.3, 7.2, 2.2)	3.67 td (7.8, 1.9)
6′	4.47 dd (11.8, 7.3) 4.63 dd (11.8, 2.1)	4.45 dd (11.8, 7.0) 4.56 dd (11.8, 2.2)	4.44 dd (11.8, 6.8) 4.55 dd (11.8, 2.1)
1″			
2″	7.25 s	7.02 s	7.06 s
3″			
4″			
5″			
6″	7.25 s	7.02 s	7.06 s
7″			
3″,5″-OCH_3	3.83 s		
4″-OCH_3		3.87 s	

a) 500 MHz. *b*) 400 MHz. δ values in ppm and coupling constants (in parentheses) in Hz.

by HMBC correlations observed between signals at δ_H 4.78 (Glc-H-1′) and δ_C 148.5 (C-5), and at δ_H 4.45 (H-6′) and δ_C 167.7 (C-7″) (Fig. 1). The anomeric configuration of the sugar was determined to be β ($J_{1',2'}$=7.7 Hz).

To determine the absolute configuration of the chiral center at C-4 in **6**—**8**, these compounds were hydrolyzed with naingenase to give the same product 4,5,8-trihydroxy-α-tetralone (**6a**, $[\alpha]_D$ +13° in EtOH), which afforded a tribenzoate (**6b**, $[\alpha]_D$ −17° in EtOH) on benzoylation. The circular dichroic (CD) spectrum of **6a** exhibited a negative Cotton effect at 265 nm ($\Delta\varepsilon$=−16.8), indicating *S*-configuration at C-4 in comparison with the reported data of natural tetralones, such as (4*R*)-shinanolone ($\Delta\varepsilon$=+3.0 at 272 nm),[30] (4*R*)-isoshinanolone ($\Delta\varepsilon$=+33.0 at 240 nm)[31] and (4*S*)-regiolone ($\Delta\varepsilon$=−44.3 at 238 nm).[32]

In compound **7**, a hydroxy group at C-4 was subsequently arranged at an α-oriented axial position on the basis of the ^{1}H-NMR spectrum, where the coupling constant of H-4 (t) was $J_{4\beta,3\alpha}$=$J_{4\beta,3\beta}$=3.3 Hz. This was further supported by the conformation of the cyclohexanone ring possessing a half-chair form (Fig. 2), which was deduced from the multiplicities of Hα-2 (axial) at δ 3.01 (ddd, $J_{2\alpha,2\beta}$=17.7 Hz, $J_{2\alpha,3\beta}$=11.8 Hz, $J_{2\alpha,3\alpha}$=6.8 Hz) and Hβ-2 (equatorial) at δ 2.48 (dt, $J_{2\beta,2\alpha}$=17.7 Hz, $J_{2\beta,3\alpha}$=$J_{2\beta,3\beta}$=3.4 Hz). The appreciable difference in chemical shifts of the above protons might be caused by environmental differences around the protons: *i.e.*, Hα-2 experiences the deshielding effects of the C=O and axial C_4-OH, whereas Hβ-2 only feels the effect of C=O. Thus, Hα-2 was more deshielded compared to Hβ-2.[32] The structure of **7** was consequently determined as 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,5″-dihydroxy-4″-methoxybenzoyl)] glucopyranoside.

Compound **8**, a light yellow amorphous powder, showed a

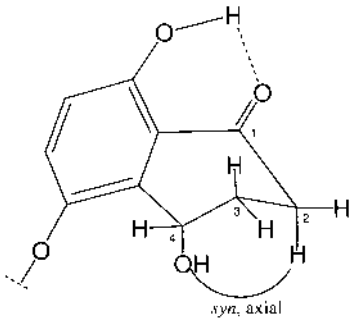

Fig. 2. Partial Structure of **7**

quasi-molecular ion peak $[M+Na]^+$ at *m/z* 531 in the positive FAB-MS spectrum. Inspection of the NMR data (Tables 2 and 3) of **8** suggested a similar structure with **7**, except the absence of a methyl group in the galloyl moiety. This was supported by the difference of 14 mass units between quasi-molecular ion peaks of **7** and **8** in the positive FAB-MS, as well as the presence of a [galloyl group]$^+$ ion peak at *m/z* 153 in **8**. The connectivities of the sugar and galloyl groups were established by the HMBC experiment, where glucose and the galloyl group were linked at C-5 and C-6′, respectively. The anomeric configuration was determined to be β ($J_{1',2'}$=7.7 Hz). The configuration of the hydroxy group at C-4 was the same as that of **7**. From these findings, the structure of **8** was established as 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,4″,5″-trihydroxybenzoyl)] glucopyranoside.

Inhibitory Effects of Compounds on RT and RNase H Activities In an effort to develop anti-AIDS agents, we have so far isolated several inhibitory substances against enzymes essential for proliferation of HIV, from medicinal plants, *i.e.*, putranjivan A from *Phyllanthus emblica*,[33] corilagin and 1,3,4,6-tetragalloylglucose from *Chamaesyce hyssopifolia*,[34] and magnesium lithospermate, calcium and magnesium rosmarinate from *Cordia spinescens*,[35] on RT (RNA-dependent DNA polymerase). However, they were not sufficiently potent for further development as anti-HIV drugs. In the course of screening for novel natural products with anti-HIV-1 RT and RNase H activities, we found that the methanol extract of *J. mandshurica* (stem-bark) appreciably inhibited both enzyme activities. The extract was further fractionated with various solvents into hexane-, ethyl acetate- and butanol-soluble fractions. Of these, the ethyl acetate-soluble fraction inhibited the HIV-RT and RNase H activities most potently with IC_{50} values of 0.047 and 22 μg/ml. From the ethyl acetate-soluble fraction, 14 compounds (**1**—**14**) were isolated as mentioned above and their inhibitory potencies were examined against both enzyme activities.

Substances that inhibit *in vitro* HIV RT are likely to fall into one of three categories; i) substances potently blocking both RT and RNase H activities; ii) those inhibiting preferably the RT activity; and iii) those selectively inhibiting the RNase H activity without any significant effect on the RT function.[36] Of the compounds isolated in this experiment, **8**, **13** and **14** belonged to the second category; they showed potent inhibition against HIV-1 RT activity with IC_{50} values of 5.8, 0.067, and 0.040 μM, respectively, but showed moderate inhibition against RNase H activity with IC_{50} values of 330, 310 and 39 μM (Fig. 3, Table 4). The inhibitory potencies of **13** and **14** against HIV-1 RT were stronger than those of cori-

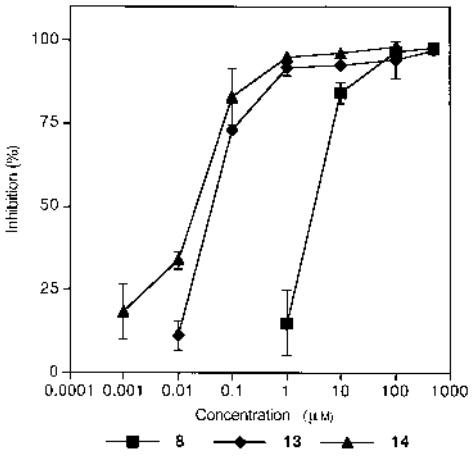

Fig. 3. Effect of **8**, **13** and **14** on RT Activity (Mean±S.D., $n=3$)

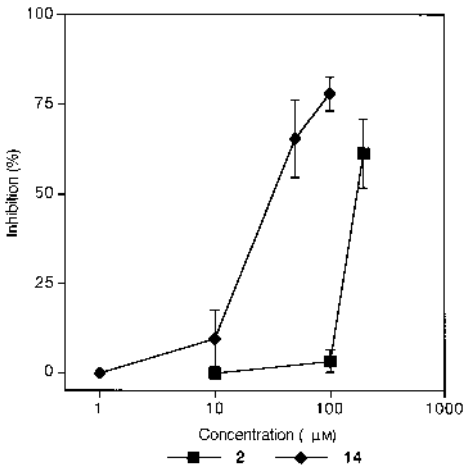

Fig. 4. Effect of **2** and **14** on RNase H Activity (Mean±S.D., $n=3$)

Table 4. Effects of Compounds Isolated from *J. mandshurica* on RT and RNase H Activities of HIV-1 RT

Compound	IC_{50} (μM)	
	RT	RNase H
1	>500	490
2	290	156
3	323	460
4	>500	>500
5	>500	>500
6	>500	>500
7	330	>500
8	5.8	330
9	>500	>500
10	>500	>500
11	>500	>500
12	>500	>500
13	0.067	310
14	0.040	39
Adriamycin[a]	45	
Illimaquinone[b]		50

a) Inhibitor of HIV-1 RT. *b*) Inhibitor of RNase H.

lagin and 1,3,4,6-tetragalloylglucose, which lack a galloyl group at C-2 and showed IC_{50} values of 20 and 86 μM, respectively, in our previous experiment,[34] a galloyl group attached at C-2 of glucopyranose seems to enhance significantly the inhibitory potency of galloylglucoses against HIV-1 RT. Compound **14** was comparable in inhibitory potency to illimaquinone with an IC_{50} of 50 μM and used as a positive control (Fig. 4, Table 4). Of the characteristic ingredients such as naphthalenyl and α-tetralonyl glycosides present in this plant, **2**, **3** and **8** showed moderate inhibition against both enzyme activities, and the inhibitory potency of **2** against RNase H activity ($IC_{50}=156$ μM) was stronger than that against RT activity ($IC_{50}=290$ μM). The inhibitory potencies of **6**, **7** and **8** against RT activity increased in this order, as represented by IC_{50} values of >500, 330 and 5.8 μM, being proportional to the number of free hydroxyls on the galloyl residue in the molecule. It has been reported that naphthalene derivatives such as naphthalenesulfonic acid[37] and *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH),[38] have anti-HIV-1 RT activity. Flavonoids, **9**—**12**, were inactive against HIV-1 RT and RNase H activities.

Experimental

Optical rotations were measured with a DIP-360 automatic polarimeter (JASCO Co., Tokyo, Japan). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan). CD spectra were recorded in EtOH on a JASCO J-715 CD dispersion spectrometer (JASCO Co.). IR spectra were measured with an FT/IR-230 infrared spectrometer (JASCO Co.). ^{1}H- and ^{13}C-NMR spectra were measured with UNITY 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz; Varian Co., Palo Alto, U.S.A.) or JEOL JNA-LAA 400 WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz; JEOL Co., Tokyo, Japan) spectrophotometers. The chemical shifts are presented as ppm with tetramethylsilane as an internal standard. FAB-MS was measured with a JEOL JMX-AX 300L spectrometer (JEOL Co.) using glycerol as a matrix. Column chromatography was carried out on silica gel (Kieselgel 60, 70—230 mesh, Merck Co., Darmstadt, Germany), Sephadex LH-20 (Pharmacia, Upsala, Sweden), Amberlite MB-3 (Rohm and Haas Co., Philadelphia U.S.A.), and ODS (Chromatorex, 100—200 mesh, Fuji Silysia Chemical Ltd., Aichi, Japan). MPLC was carried out on LiChroprep RP-18 (size A, Merck Co.). TLC was carried out on pre-coated silica gel 60 F_{254} plates (0.25 mm, Merck Co.), and spots were detected under UV light and by 10% H_2SO_4 followed by heating.

Plant Materials The stem-bark of *J. mandshurica* was collected during September 1998 at a mountain area of Kimchun, Kyungbook, Korea, and dried at room temperature for 3 weeks. A voucher specimen is deposited at the herbarium of the College of Pharmacy, Chungnam National University, Taejon, Korea.

Isolation Procedure The stem-bark of *J. mandshurica* (3.0 kg) was extracted with MeOH (6000 ml×3) at room temperature for 24 h to give an extract (390 g). A part of the MeOH extract (300 g) was suspended in H_2O (2500 ml) and extracted with hexane (2500 ml×3) to give a hexane-soluble fraction (48 g). The resulting H_2O layer was extracted with CH_2Cl_2 (3000 ml×3), EtOAc (3000 ml×3) and BuOH (3000 ml×3), successively. The EtOAc-soluble fraction (90 g) was chromatographed on a column of silica gel (1 kg). The column was eluted using a stepwise gradient of $CHCl_3$, MeOH and H_2O to give 6 fractions (Fr. A—F; 2.7, 15.9, 23.9, 7.5, 5.4 and 10.2 g, respectively). Repeated column chromatography of Fr. B on silica gel ($CHCl_3$–MeOH, 9 : 1), Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 9) and reversed phase ODS (50% aq. MeOH), followed by MPLC on RP-18 (50% aq. MeOH and 70% aq. CH_3CN) afforded **3** (60 mg), **4** (15 mg), **6** (335 mg), **7** (3.3 mg), **9** (165 mg) and **10** (189 mg). Repeated column chromatography of Fr. C on Sephadex LH-20 (MeOH and $CHCl_3$–MeOH, 1 : 9), silica gel ($CHCl_3$–MeOH, 8 : 2) and reversed phase ODS (40% aq. MeOH), followed by MPLC on RP-18 (40% aq. MeOH and 80% aq. CH_3CN) furnished **1** (18 mg), **2** (5 mg), **5** (164 mg), **8** (11 mg), **11** (168 mg), **12** (345 mg), **13** (340 mg) and **14** (294 mg).

1,4,8-Trihydroxynaphthalene 1-*O*-[α-L-Arabinofuranosyl-(1→6)-β-D-glucopyranoside] (1) Light brown amorphous powder, $[\alpha]_D$ −94° ($c=0.1$, MeOH). IR ν_{max} cm^{-1}: 3400, 1610, 1520, 1405, 1375, 1255, 1165. UV λ_{max} nm (log ε): 224 (4.6), 306 (3.8), 326 (3.7), 342 (3.7). Positive FAB-MS *m/z*: 493 $[M+Na]^+$, 471 $[M+H]^+$. ^{1}H- and ^{13}C-NMR data: see Tables 1 and 2.

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-Glucopyranoside (2)[21] Brown amorphous powder, $[\alpha]_D$ −85° ($c=0.1$, MeOH). IR ν_{max} cm^{-1}: 3400, 1615, 1520, 1405, 1375, 1260, 1070. UV λ_{max} nm (log ε): 224 (4.6), 306 (4.2), 326

(4.1), 342 (4.1).

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(4″-Hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (3)[19] Light brown amorphous powder, $[\alpha]_D$ −49° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1680, 1610, 1520, 1460, 1420, 1340, 1215, 1120. UV λ_{max} nm (log ε): 224 (4.8), 288 (4.1), 326 (4.1), 342 (3.8).

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,5″-Dihydroxy-4″-methoxybenzoyl)]glucopyranoside (4) Light brown amorphous powder, $[\alpha]_D$ −49° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1610, 1520, 1360, 1240, 1160. UV λ_{max} nm (log ε): 224 (4.7), 265 (3.9), 305 (3.9), 326 (3.8), 342 (3.8). Positive FAB-MS *m/z*: 527 $[M+Na]^+$, 505 $[M+H]^+$. ^{1}H- and ^{13}C-NMR data : see Tables 1 and 2.

1,4,8-Trihydroxynaphthalene 1-*O*-β-D-[6′-*O*-(3″,4″,5″-Trihydroxybenzoyl)]glucopyranoside (5)[19] Brown needles (MeOH–H_2O), $[\alpha]_D$ −53° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1690, 1610, 1520, 1445, 1350, 1070, 1030. UV λ_{max} nm (log ε): 224 (4.8), 282 (4.1), 326 (3.9), 342 (3.9).

4α,5,8-Trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(4″-Hydroxy-3″,5″-dimethoxybenzoyl)]glucopyranoside (6)[20] Light yellow amorphous powder, $[\alpha]_D$ −53° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1705, 1645, 1610, 1515, 1465, 1410, 1335, 1225, 1115, 1070. UV λ_{max} nm (log ε): 216 (4.5), 262 (4.1), 348 (3.5).

4α,5,8-Trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,5″-Dihydroxy-4″-methoxybenzoyl)]glucopyranoside (7) Light yellow amorphous powder, $[\alpha]_D$ −18° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1640, 1600, 1470, 1440, 1360, 1250, 1160. UV λ_{max} nm (log ε): 214 (4.5), 260 (4.1), 348 (3.3). Positive FAB-MS *m/z*: 545 $[M+Na]^+$, 523 $[M+H]^+$. ^{1}H- and ^{13}C-NMR data: see Tables 3 and 2.

4α,5,8-Trihydroxy-α-tetralone 5-*O*-β-D-[6′-*O*-(3″,4″,5″-Trihydroxybenzoyl)]glucopyranoside (8) Light yellow amorphous powder, $[\alpha]_D$ −34° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1640, 1610, 1470, 1350, 1340, 1230, 1070. UV λ_{max} nm (log ε): 216 (4.6), 262 (4.1), 350 (3.5). Positive FAB-MS *m/z*: 531 $[M+Na]^+$, 509 $[M+H]^+$. ^{1}H- and ^{13}C-NMR data: see Tables 3 and 2.

Taxifolin (5,7,3′,4′-Tetrahydroxyflavanol, 9)[22] White amorphous power, $[\alpha]_D$ +20° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1620, 1520, 1470, 1360, 1265, 1165. UV λ_{max} nm (log ε): 288 (4.2), 328 (sh).

Afzelin (Kaempferol 3-*O*-α-L-Rhamnopyranoside, 10)[23] Yellow amorphous power, $[\alpha]_D$ −184° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3280, 1655, 1615, 1500, 1450, 1365, 1070, 1060. UV λ_{max} nm (log ε): 264 (4.3), 342 (4.1).

Quercitrin (Quercetin 3-*O*-α-L-Rhamnopyranoside, 11)[24] Yellow amorphous power, $[\alpha]_D$ −178° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3320, 1660, 1610, 1500, 1450, 1360, 1300, 1200. UV λ_{max} nm (log ε): 254 (4.2), 314 (sh), 350 (4.1).

Myricitrin (Myricetin 3-*O*-α-L-Rhamnopyranoside, 12)[24] Yellow amorphous power, $[\alpha]_D$ −141° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3270, 1655, 1610, 1500, 1455, 1340, 1290. UV λ_{max} nm (log ε): 254 (4.2), 312 (sh), 354 (4.1).

1,2,6-Trigalloylglucopyranose (13)[25] White amorphous power, $[\alpha]_D$ −94° (*c*=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1710, 1610, 1540, 1525, 1450, 1355, 1310, 1240, 1210. UV λ_{max} nm (log ε): 216 (4.6), 278 (4.4).

1,2,3,6-Tertagalloylglucopyranose (14)[26] White amorphous power, $[\alpha]_D$ +39° (*c*=0.1, MeOH). 3400, 1700, 1610, 1540, 1455, 1355,1200. UV λ_{max} nm (log ε): 216 (4.9), 278 (4.5).

Determination of Sugars in 1, 4, 7 and 8 Each sample (2 mg) was refluxed with 4 N HCl–dioxane (1 : 1, 2 ml) for 2 h. The mixture was extracted with EtOAc (5 ml×3). The residual water layer was neutralized with Amberlite MB-3 and dried to give a residue. The residue was dissolved in pyridine (1 ml), to which 0.1 M L-cysteine methyl ester hydrochloride in pyridine (2 ml) was added. The mixture was kept at 60 °C for 1.5 h. After the reaction mixture was dried *in vacuo*, the residue was trimethylsilylated with hexamethyldisilazane-trimethylchlorosilane (0.1 ml) at 60 °C for 1 h. The mixture was partitioned between hexane and H_2O (0.3 ml each) and the hexane extract was analyzed by gas-liquid chromatography (GLC). In the acid hydrolysate of **1**, D-glucose and L-arabinose were confirmed by comparison of the retention times of their derivatives with those of D-glucose, L-glucose, D-arabinose and L-arabinose derivatives prepared in a similar way, which showed retention times of 21.34, 22.00, 17.37 and 16.34 min, respectively.

The sugars from acid hydrolysis of **4**, **7** and **8** were identified by TLC on silica gel with a solvent system of EtOAc–MeOH–H_2O–AcOH (65 : 20 : 15 : 15). The spots on the plate were visualized by spraying an anisaldehyde-H_2SO_4 solution.

Enzymatic Hydrolysis of 6—8 Naringinase (50 mg, Sigma Co.) was added to a suspension of **6**, **7** or **8** (5—10 mg) in 50 mM acetate buffer (pH 5.5) and the mixture was stirred at 37 °C for 5 h. The reaction mixture was extracted with EtOAc (10 ml×3) and evaporated to dryness. The residue was chromatographed on silica gel eluting with hexane–acetone (2 : 1) to give 4,5,8-trihydroxy-α-tetralone (**6a**) in 50—60% yields. Compound **6a**, yellow amorphous powder, $[\alpha]_D^{26}$ +13° (*c*=0.1, EtOH). UV λ_{max} nm (log ε): 235 (4.0), 265 (3.7), 372 (3.5). CD ($c=1.55\times10^{-2}$) Δε (nm): −16.79 (265), −5.79 (319). ^{1}H-NMR ($CDCl_3$+CD_3OD) δ: 2.60 (ddd, *J*=17.3, 10.3, 4.7 Hz, Hβ-2), 2.90 (ddd, *J*=17.3, 7.1, 4.5 Hz, Hα-2), 2.33 (m, Hβ-3), 2.20 (m, Hα-3), 5.21 (dd, *J*=8.1, 4.5 Hz, H-4), 7.07 (d, *J*=9.3 Hz, H-6), 6.81 (d, *J*=9.3 Hz, H-7). ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 204.4 (C-1), 35.2 (C-2), 30.8 (C-3), 65.5 (C-4), 147.4 (C-5), 126.3 (C-6), 118.0 (C-7), 156.0 (C-8), 115.2 (C-9), 128.0 (C-10).

Benzoylation of 6a Benzoyl chloride (100 mg) was added to a solution of **6a** (2.5 mg) in pyridine (0.1 ml) and the reaction mixture was kept overnight at room temperature. The mixture was evaporated to give a residue, which was purified by preparative thin layer chromatography on silica gel with hexane–acetone (3 : 1) to give 4,5,8-tribenzoyloxy-α-tetralone (2.6 mg, **6b**); white amorphous powder, $[\alpha]_D^{26}$ −17 (*c*=0.1, EtOH). UV λ_{max} nm (log ε): 235 (4.0). CD ($c=0.59\times10^{-2}$) Δε (nm): −2.35 (254), −3.24 (280). ^{1}H-NMR ($CDCl_3$) δ: 2.61 (dt, *J*=16.8, 3.6 Hz, Hβ-2), 2.98 (ddd, *J*=16.8, 13.1, 5.6 Hz, Hα-2), 2.48 (2H, m), 6.61 (t, *J*=3.3 Hz, H-4), 7.37 (5H, m), 7.57 (5H, m), 7.66 (1H, m), 7.86 (2H, m), 7.99 (2H, m), 8.26 (2H, m). ^{13}C-NMR ($CDCl_3$) δ: 195.5 (C-1), 34.5 (C-2), 27.1 (C-3), 64.5 (C-4), 146.9 (C-5), 164.9, 165.7, 169.2 (3×benzoyl group C=O).

RNase H Activity Assay For the assay of HIV-1-RT-associated RNase H,[4] HIV-1 RT was adjusted to 2.5 U/μl with a solution of 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$ and 2.5 mM dithiothreitol (DTT). A mixture (20 μl) containing of 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 2.5 mM DTT, 7.2 nM of [^{3}H]poly(rA)·poly(dT) (370 kBq/ml), and a test sample in dimethyl sulfoxide (DMSO; final concentration of 5%) was preincubated at 37 °C for 5 min. The reaction mixture was kept for 2 h at 37 °C. A blank reaction was carried out under the same conditions without adding enzyme, and a control reaction was done in the absence of a test sample. The reaction was terminated by addition of 20 μl of 0.02 M EDTA. The mixture was applied onto a Whatman DE81 paper disc, which was washed batchwise with 3 ml of 5% Na_2HPO_4, distilled water three times, ethanol once and ether once. The paper disc was then dried and immersed in 3 ml of scintillation fluid. The RNase H activity was measured as the inhibition of the degradation of RNA in a hybrid in the presence of a test sample as follows:

$$\text{inhibition (\%)}=[1-(dpm_{blank}-dpm_{sample})/(dpm_{blank}-dpm_{contl.})]\times100$$

Illimaquinone was used as a positive control and inhibited RNase H activity with an IC_{50} of 50 μM under the above conditions.

DNA–RNA Hybrid Preparation 0.57 nM poly(dT) was annealed to 0.32 nM poly(rA) and 5 pM [^{3}H]poly(rA) in 50 μl of H_2O. The mixture was heated to 90 °C for 5 min, allowed to cool gradually to 37 °C, incubated for 30 min, kept at room temperature for 30 min and finally stored at −20 °C.[39]

RT Activity Assay For the assay of RT,[40,41] HIV-1 RT was adjusted to 0.01 U/μl with a solution of 0.2 M phosphate buffer (pH 7.2), 50% glycerol, 2 mM DTT and 0.02% of Triton X-100. The reaction mixture (20 μl) containing 50 mM Tris–HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 1.25 μg/ml (*ca.* 16 nM) poly(rA)·oligo$(dT)_{12-18}$ as a template-primer, 250 nM dTTP, 100 nM [*methyl*-^{3}H]dTTP (18.5 MBq/ml) and 0.01 U/μl of RT, and 1.0 μl of a test sample dissolved in DMSO (final concentration of 5%) was incubated at 37 °C for 1 h. A control reaction was done under the same conditions without adding a test sample. The reaction was terminated by addition of 20 μl of 0.02 M EDTA. The resulting mixture was applied onto Whatman DE81 paper disc and washed in a similar manner as described above. The paper disc was then dried and immersed in 3 ml of scintillation fluid. The amount of a polymer fraction was determined by counting the radioactivity on the paper disc according to the incorporation of ^{3}H-labeled substrate into the DNA polymer. The calculation of the inhibitory potency for the test sample was done as follows:

$$\text{inhibition (\%)}=[1-(dpm_{comp.}/dpm_{contl.})]\times100$$

Adriamycin was employed as a positive control and inhibited the RT with an IC_{50} of 46 μM under the above conditions.

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