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# ANTI-AIDS AGENTS, 2[1]: INHIBITORY EFFECTS OF TANNINS ON HIV REVERSE TRANSCRIPTASE AND HIV REPLICATION IN H9 LYMPHOCYTE CELLS

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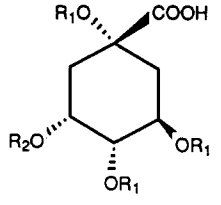
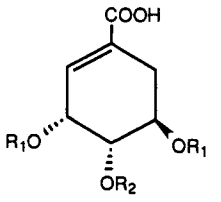
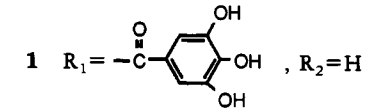
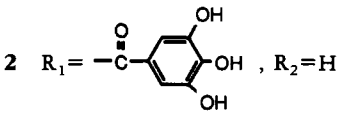
ABSTRACT.—Nine tannins, including gallo- and ellagitannins, were evaluated as potential inhibitors of HIV replication. 1,3,4-Tri-*O*-galloylquinic acid [**1**], 3,5-di-*O*-galloylshikimic acid [**2**], 3,4,5-tri-*O*-galloylshikimic acid [**3**], punicalin [**6**], and punicalagin [**7**] inhibited HIV replication in infected H9 lymphocytes with little cytotoxicity. Two compounds, punicalin and punicacortein C [**8**], inhibited purified HIV reverse transcriptase with $ID_{50}$ of 8 and 5 μM, respectively. Further studies with H9 lymphocytes indicated that chebulagic acid [**5**] and punicalin did not inactivate virus directly. However, 1,3,4-tri-*O*-galloylquinic acid and 3,5-di-*O*-galloylshikimic acid were more effective inhibitors under those conditions. All tannins appear to inhibit virus–cell interactions. Thus, inspite of their anti-RT activity, the mechanism by which tannins inhibit HIV may not be associated with this enzyme.

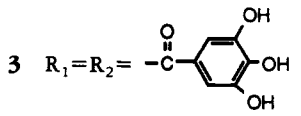
Recently, much progress has been made in the study of human immunodeficiency virus [HIV-I(HTLV-III/LAV)], the causative agent of acquired immunodeficiency syndrome (AIDS), including the elucidation of the genomic structure of HIV as well as the mechanism of HIV infection (1–4). Because reverse transcriptase (RT) plays a very important role in controlling the replication of the HIV, RT is one of the most attractive targets for the development of anti-HIV drugs. Among the many drugs tested, phosphonoformate (PFA), 3′-azido-2′,3′-di-deoxythymidine (AZT), and 2′,3′-dideoxycytidine (DDC) were shown to be useful in the treatment of AIDS patients, and the selective antiviral action of some of these drugs was suggested to be associated with




[1]For Part 1, see Nishizawa *et al.* (5).

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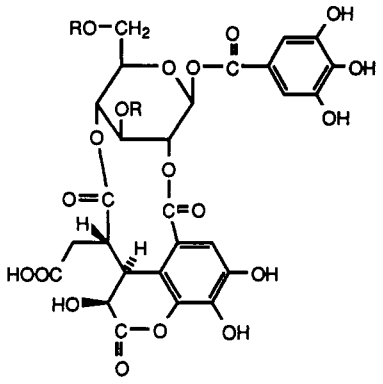
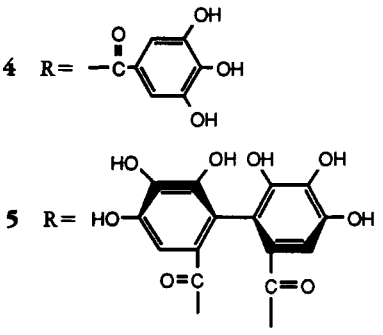




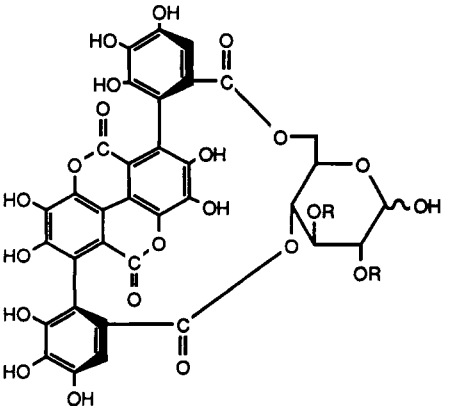
their unique interaction with RT (2). The use of HIV-RT in vitro for detecting potential chemotherapeutic agents against HIV is a logical approach.

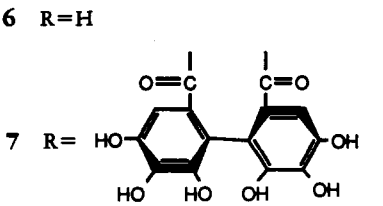
In a previous paper (5), we demonstrated that galloylquinic acids, especially tetragalloylquinic acids, are potent HIV-RT inhibitors. Because the structures of these galloylquinic acids are quite different from those of known HIV inhibitors mentioned above, other classes of tannins have been investigated for their inhibitory effect on HIV replication in cell culture and HIV-RT.

## RESULTS AND DISCUSSION

Tannins have been screened as inhibitors of HIV replication in H9 lymphocytes. As demonstrated in Figures 1 and 2, eight tannins showed significant anti-HIV activity (greater than 50% inhibition of virus replication) at concentrations that do not inhibit H9 cell growth. Punicalin [**6**] showed less than 15% growth inhibition even at 30 μM (see Figure 2). One compound, sanguiin H-11 [**9**], did not significantly inhibit HIV replication in H9 cells (data not shown). The tannins examined included gallotannins and ellagitannins. The gallotannins contained a quinic acid core (compound **1**) or a shikimic acid core (**2** and **3**). The ellagitannins contained a modified hexahydroxydiphenoyl moiety and/or a related acyl group (**4**–**7**) or an open chain glucose core (**8**). Compound **9** was an ellagitannin tetramer.

In order to investigate the mechanism of HIV inhibition, tannins were also evaluated as inhibitors of purified HIV-RT. Three compounds, sanguiin H-11 [**9**], punicalin [**6**], and punicacortein C [**8**] were potent inhibitors of HIV-RT with $ID_{50}$ of 20, 8, and 5 μM, respectively. The most potent RT inhibitors, **6** and **8**, contain a gal-





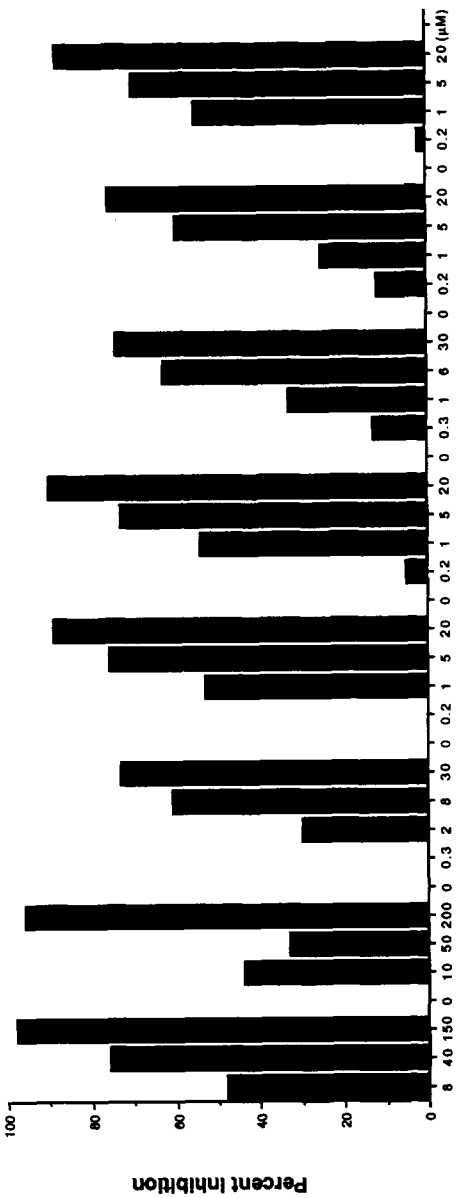



FIGURE 1. Anti-HIV effect of tannins **1**–**8**. This represents a normalized percent HIV replication inhibition as measured by p24 antigen capture. This graph is data from one of two experiments which showed similar results.

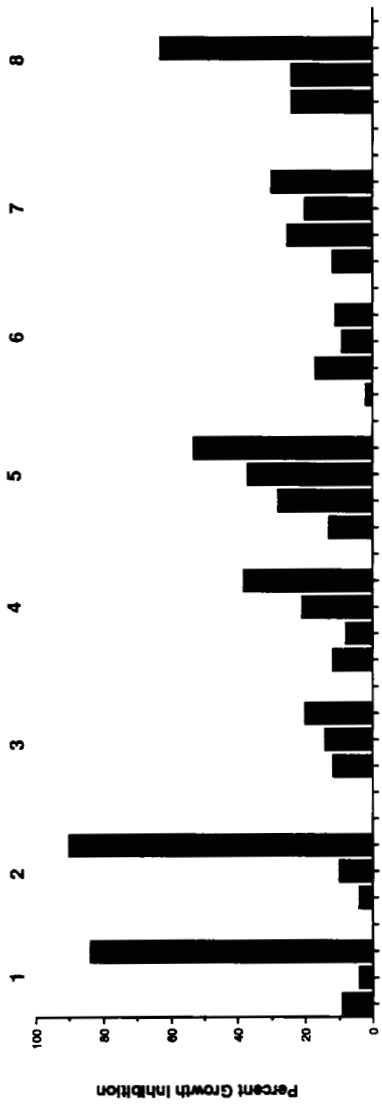
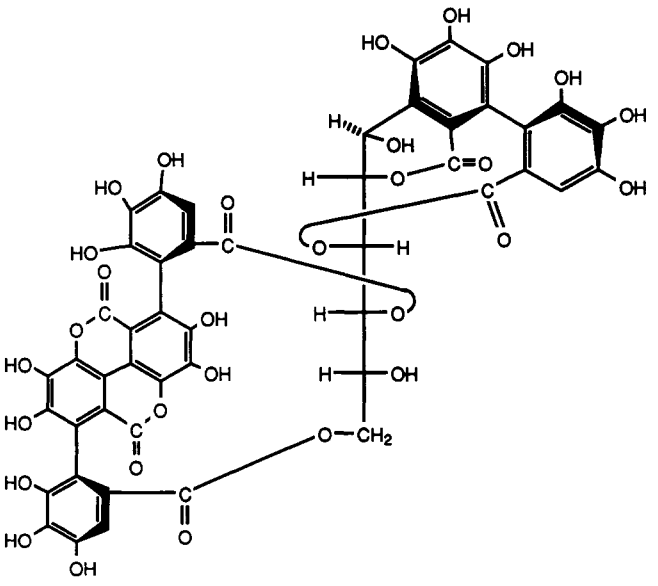



FIGURE 2. Effect of tannins **1–8** on H9 cell growth. This represents the percent growth inhibition of uninfected H-9 cells treated with different tannins.



**8**

lagyl moiety, suggesting that this structure may be important for RT inhibition. The RT inhibition by **6** is concentration-dependent, and the reaction catalyzed by RT in the presence of drug is linear with time (Figures 3 and 4). The degree of inhibition by **6** and **8** varied with the concentration of substrates in the reaction mixture (Table 1). This could be due to the interaction of the tannins with DNA. DNA binding was measured by the ethidium bromide displacement assay (Figure 5). Compounds **6** and **8** were about sixty-fivefold less active in this assay than actinomycin D.

Further investigations of the mechanism of HIV inhibition were conducted in cell culture. The tannins were added to virions before infection, during the infection step,

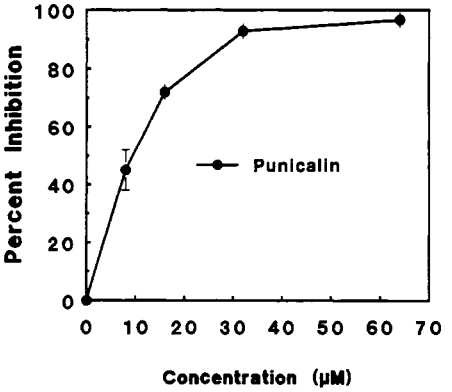



FIGURE 3. Effect of punicalin [**6**] on HIV-RT. Different amounts of punicalin were incubated in the standard RT mixture for 60 min. After washing and counting, the drug-treated samples were compared to drug-free controls.

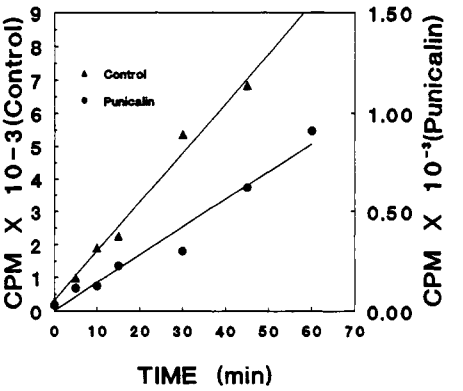



FIGURE 4. Time course in the presence of punicalin [**6**] at 12.5 μM. Drug was incubated in the standard assay mixture for various times. After washing and counting, samples were compared to drug-free controls.

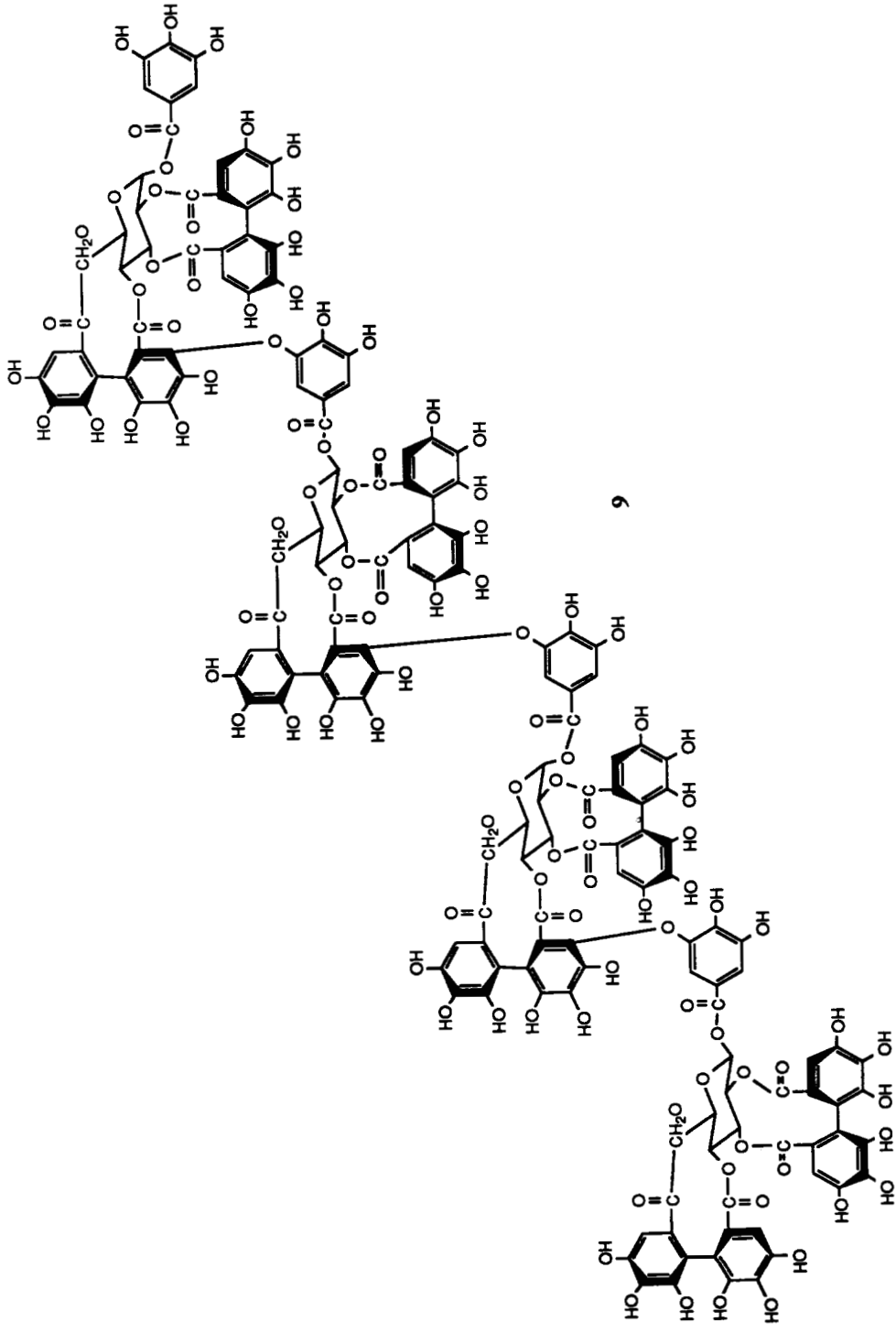


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TABLE 1. Effect of Tannins on HIV Polymerase.[a]

| Varied Components | Assay Mixtures | | |
|---|---|---|---|
| | A | B | C |
| Activated DNA (μg/ml) | 75 | 75 | 375 |
| dTTP (μM) | 5 | 25 | 5 |
| | Percent Activity (mean ± SD) | | |
| Punicalin [**6**] (64 μM) | 11.4 ± 2 | 34.4 ± 7 | 45.3 ± 8 |
| Punicacortein C [**8**] (46 μM) | 21.5 ± 3 | 37.6 ± 7 | 46.2 ± 4 |

[a]Punicalin and punicacortein C were incubated in three reaction conditions as indicated. The rest of the condition is the same as the regular DNA reaction mixture described below: each condition in a 50-μl volume contained 50 mM Tris HCl pH 8.0, 8 mM $MgCl_2$, 100 μg/ml BSA, 100 μM each of dATP, dGTP, and dCTP, and 1 mM DTT. The reactions were incubated 30 min. at 37°. The rest of the conditions are as in the RT assay.

or immediately after infection. Figure 6 shows that the degree of HIV inhibition depends not only on the compound tested but also on when the tannin is present in the inhibition assay. Note that although the concentrations of the tannins tested in this experiment varied somewhat (9.2–21 μM), the extent of HIV inhibition by tannins added after infection (our normal protocol) was similar (35–65%). Preincubation of virus with two tannins, chebulagic acid [**5**] and punicalin [**6**], did not inhibit HIV replication significantly. However, when five other tannins, 1,3,4-tri-*O*-galloylquinic acid [**1**], 3,5-di-*O*-galloylshikimic acid [**2**], chebulinic acid [**4**], punicalagin [**7**], and punicacortein C [**8**], were preincubated with virions before infection, all inhibited HIV. Two of these (**1** and **2**) were more effective inhibitors when virions were preincubated with tannins than when the tannins were added to the culture post-infection. All tannins virtually eliminated HIV replication when present during the infection step. These results suggest that the tannins might inhibit virus–cell interactions. The tannins that inhibit after preincubation with virions (e.g., **1** and **2**) may bind tightly to virions, inactivating them and preventing infection. The two compounds that do not inhibit after preincubation (**5** and **6**) may interact only weakly with virions, so that the

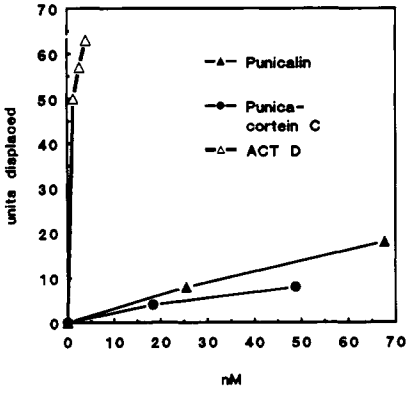


FIGURE 5. Ethidium displacement by punicalin [**6**]. The procedure is described in the Experimental section. Actinomycin D was used as a positive control.

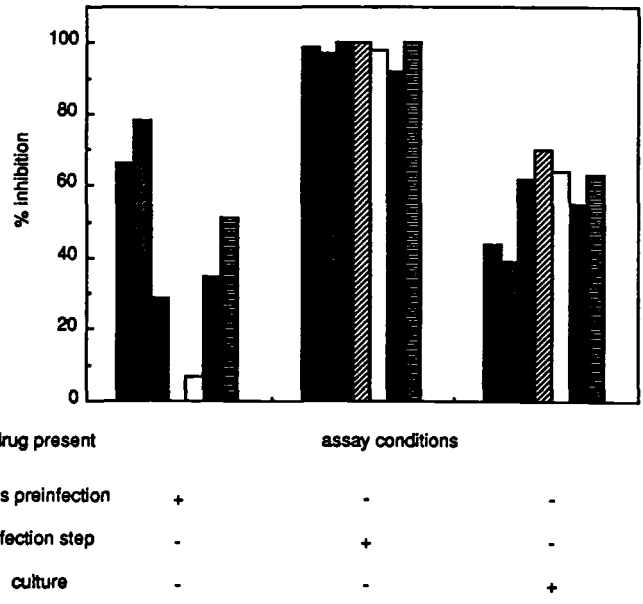


FIGURE 6. Effect of addition of tannins to cultures at different times. Tannins were assayed for anti-HIV activity under different assay conditions (see Experimental). Tannins were all assayed at 10 μg/ml. Compounds tested and their molar concentrations are: **1** (18 μM) ■; **2** (21 μM) ▨; **4** (10 μM) ▪; **5** (10 μM) ▨; **6** (13 μM) □; **7** (9.2 μM) ■; and **8** (9.2 μM) ▤.

tenfold dilution of treated virions prior to the infection step may decrease the virus–tannin interaction. They may still interfere with virus binding to H9 cells when present in the virus-cell mixture at 10 μg/ml.

In summary, our results suggest that a variety of tannins are capable of inhibiting purified HIV-RT. They may also inhibit HIV replication in cell culture by additional or other mechanisms, probably by interfering with HIV–cell interactions. We are presently characterizing the nature of the interactions between tannins, HIV, and H9 cells.

## EXPERIMENTAL

MATERIAL.—The nine tannin samples used for this investigation were isolated from the plant materials as reported previously: 1,3,4-tri-*O*-galloylquinic acid [**1**] (6), 3,5-di-*O*-galloylshikimic acid [**2**] (7), 3,4,5-tri-*O*-galloylshikimic acid [**3**][5], chebulinic acid [**4**] (8), chebulagic acid [**5**] (9), punicalin [**6**] (10), punicalagin [**7**] (10), punicacortein C [**8**] (11), and sanguiin H-11 [**9**] (12).

HIV-RT ASSAY.—The HIV-RT assay was performed according to the method described by Cheng *et al.* (4). The immuno-affinity purified enzyme used was isolated from virions released by human T cells infected with HIV. Poly rA oligo $dT_{10}$ from Pharmacia (Piscataway, New Jersey) was used as the template to measure the incorporation of [$^3$H] dTMP (20 μM). The percentage of inhibition was determined by comparing the RT activity of tannin-containing assays to that of the drug-free controls.

HIV INHIBITION ASSAY.—Our standard HIV inhibition assay was performed by incubating H9 lymphocytes ($3.5 \times 10^6$ cells/ml) in the presence or absence of HIV-1 (HTLV-IIIB) for 1 h at 37°. Cells were washed thoroughly to remove unabsorbed virions and resuspended at $4 \times 10^5$ cells/ml in culture medium. Aliquots (1 ml) were placed in wells of 24-well culture plates containing an equal volume of medium and an equal concentration of test compound (diluted in culture medium). After incubation for 3

[5]G. Nonaka, unpublished data.

days at 37°, the cell density of uninfected cultures was determined by cell counts to assess toxicity of the test compound. A p24 antigen capture assay was used to determine the level of virus released into the medium of HIV cultures. The ability of compounds to inhibit HIV replication was measured at four different concentrations of test compound. Test compounds were considered to have anti-HIV activity if p24 levels were <70% of untreated culture (>30% inhibition). The effect of adding compounds to the inhibition assay at different times was also measured. This inhibition assay was conducted as the standard method described above, with the following modifications. Virions were preincubated with 9.2–21 μM of tannins for 1 h at 37° and then diluted tenfold into a suspension of H9 cells for infection. The infection step, using untreated virions, was conducted with tannin included (at 9.2–21 μM). Following infection and washing, the infected cultures were incubated in the presence of tannin (9.2–21 μM) for 3 days at 37° (see above-mentioned normal inhibition assay). For each assay, compound was added to the assay only at the indicated time.

ETHIDIUM DISPLACEMENT ASSAY.—This assay was performed using an Aminco fluorocolorimeter. Three ml of a solution containing 2.5 μM ethidium bromide, 2 mM Hepes, 9.4 mM NaCl, and 20 mM EDTA was mixed with 20 μl of 300 μM calf thymus DNA (Sigma D1501). This solution was placed into the fluorocolorimeter, and it was adjusted to 100% relative intensity. Then additions of the drug in DMSO or DMSO alone were added to the tube. A relative intensity reading was taken after every addition. A change of one on this scale was considered one unit of ethidium displacement (13–15).

## ACKNOWLEDGMENTS

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