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Comparative in vitro effects of AZT and extracts of *Ocimum gratissimum*, *Ficus polita*, *Clausena anisata*, *Alchornea cordifolia*, and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections

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Abstract

The effects of *Ocimum gratissimum* (GHX-2), *Ficus polita* (GHX-6), *Clausena anisata* (GHX-7), *Alchornea cordifolia* (GHX-26), *Elaeophorbia drupifera* (GHX-27), and AZT on in vitro HIV-1 and HIV-2 replication and cytopathicity were compared. All plant extracts inhibited HIV-1 strain $HTLVIII_B$ cytopathicity, the leaves of GHX-2 and the seeds of GHX-26 having high antiviral indices (110 and 90, respectively). Against HIV-2 strain GH1, the EC_{50} values ranged from <0.005 to 0.075 mg/ml when treatment was started at 40 min after virus adsorption, except for GHX-7 which showed only moderate activity and GHX-26 which had no activity. When treatment was delayed for 2 h, the plant extracts, unlike AZT, were still very effective against HIV-2. Likewise, only the plant extracts were able to attain EC_{90} values when high multiplicity of infection (MOI) with HIV-1 strain GH3 was used when treatment was delayed for 2 h. In Molt-4 cocultures with Molt-4/HIV, early cytopathic effect (CPE) of cell fusion was unaffected by AZT but was completely inhibited by all plants at noncytotoxic concentrations. In addition, GHX-27 was selectively toxic to Molt-4/HIV cells. The plant extracts also inhibited HIV-1 reverse transcriptase (RT) activity at EC_{50} values of <0.01–0.03 mg/ml. HIV-1 proviral DNA copying as determined in a polymerase chain reaction, was completely inhibited by GHX-2 and GHX-6 at 0.011 and 0.015 mg/ml, respectively. GHX-26 and GHX-27 showed only very moderate activity.

Keywords: Medicinal plants; AZT; HIV-1; HIV-2 infections

1. Introduction

Latent HIV infection in helper T lymphocytes, macrophages, and monocytes, abound throughout the lymphoid system at all stages of infection (Embretson et al., 1993). In addition, there is active and progressive HIV infection in lymphoid tissues from early to late stages of the infection (Pantaleo et al., 1993). Active and progressive HIV infections may be acute or chronic in nature. Chronically infected cells live far longer than acutely infected cells and serve as factories for virus production to attack uninfected cells. Latently infected cells are reservoirs that can be recruited to be active and progressive (Coffin, 1995). So far, no approach has been developed to tackle latently infected cells. Several nucleoside analogs including 3-azido-2′,3′-dideoxythymidine (AZT) have been developed to inhibit virus production and cytopathicity in acutely infected cells (Tuazon and Labriola, 1987; Fischl et al., 1987; Yarchoan et al., 1988). The nucleoside analogs act by inhibiting HIV-specified reverse transcriptase (RT; Tuazon and Labriola, 1987; Coffin, 1990). Rapid mutations engendering drug resistance do occur in *HIV RT* gene in the presence or absence of nucleoside analogs (Mohri et al., 1993). Protease inhibitors have also been shown to inhibit HIV production from chronically infected cells (Erickson et al., 1990; Kempf et al., 1990, 1991; Kort et al., 1993). Unfortunately, poor water solubility of the early symmetry-based HIV protease inhibitors (Erickson et al., 1990; Kempf et al., 1990), poor oral bioavailability of terminal residue-substituted new compounds (Kempf et al., 1991), as well as the presence of protease inhibitor-resistant variants of HIV in untreated and treated patients (Condra et al., 1995; Lech et al., 1996) have made it necessary to search for other drugs with mechanisms of action that may differ from those of classical nucleoside analogs and protease inhibitors. Particularly, plant products have now attracted attention as possible anti-HIV drugs (Hudson and

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Towers, 1991; Lai et al., 1990; Balzarini et al., 1992; Skinner and Ezra, 1993; Ho and Li, 1993; Aruoma et al., 1996; Yamasaki et al., 1998; Vlietinck et al., 1998; Matthee et al., 1999; Min et al., 2001). We have now studied the in vitro anti-HIV activities of five plants found in Ghana.

2. Materials and methods

2.1. Drugs and plant extracts

AZT was purchased from Sigma, St. Louis, MO, USA. *Ocimum gratissimum* (GHX-2) and *Clausena anisata* (GHX-7) were collected based on their traditional uses against herpes zoster and herpes simplex virus infections. *Ficus polita* (GHX-6), *Alchornea cordifolia* (GHX-26), and *Elaeophorbia drupifera* (GHX-27) were randomly selected for testing. Twenty-five other plants were tested but these proved negative for anti-HIV activities. All plants were collected between March and April in the southern part of Ghana and were identified by D.K. Abbiw, Director of the Herbarium, Department of Botany, University of Ghana, Legon, Ghana. Aqueous extracts were prepared by boiling cut leaves (L), seeds (S), fruits (F), stems (ST), barks (B), and roots (R) in distilled water for about 10 min in conical flasks. The water extracts were centrifuged at 1500 rpm for 10 min and the supernatants were filtered through Whatman filter papers (Whatman Laboratory Division, Springfield Mill, UK). The filtrates were then freeze-dried.

2.2. Cell cultures and viruses

Molt-4 clone 8 and M8166 cells and HIV-1 strain $HTLVIII_B$ were provided by Professor M. Hayami, Institute for Virus Research, Kyoto University, Japan. HIV-1[GH3] was isolated from a Ghanaian AIDS patient (unpublished results). HIV-2[GH1] was isolated from a Ghanaian AIDS patient who had practiced prostitution in the Ivory Coast (Ishikawa et al., 1988). The chronically HIV-1 ($HTLVIII_B$)-infected Molt-4 cell line (Molt-4/HIV) was cloned in this laboratory. Cell medium and culture conditions were similar to previous work (Ayisi et al., 1991; Ayisi, 1995).

2.3. Antiviral and cytotoxicity assays in acute infection

The modified tetrazolium-based (MTT-based) colorimetric method was used to determine the susceptibility of HIV to inhibition by drugs. Drug controls included in the assays were used to determine the cytotoxicities of drugs. The method was similar to what is already published (Ayisi et al., 1991; Ayisi, 1995, 1998). In Molt-4 and M8166 cells, 50 μl of virus and 50 μl of 1.6×10^6 cells per milliliter were added to appropriate wells of poly-L-lysine coated 96-well microtitre plates. After various periods of virus infection, 100 μl of plant extracts or drug were added. After 5 days of incubation, the MTT-based assay was performed and the 50 and 90% effective concentrations (EC_{50} and EC_{90}) as well as 50% cytotoxic concentration (CC_{50}) were determined as previously published (Ayisi et al., 1991). Each experiment contained three replicates per treatment and was done twice. Plant extracts with anti-HIV activities were then selected and the experiments repeated two more times. The values given in this paper are, therefore, the averages of four determinations.

2.4. Inhibition of Molt-4 and Molt-4/HIV cell replication

Molt-4 and Molt-4/HIV cells were washed twice with growth medium. The cells were then counted by the trypan blue exclusion method and 4 ml of 3×10^5 of each cell type per milliliter were added to wells of six-well plates containing various concentrations of plant extracts. After 2 and 4 days of incubation, the cells were recounted by the trypan blue exclusion method.

2.5. Molt-4–Molt-4/HIV cocultures

Uninfected Molt-4 cells and Molt-4/HIV cells were cocultured in the presence of plant extracts or AZT in 96-well microtitre plates. Briefly, the cells were washed twice in growth medium and counted. They were then mixed at a ratio of 1:10 (infected:uninfected), and 100 μl of 3×10^5 mixed cells per milliliter were added to the respective wells. Corresponding drug control wells contained drugs and uninfected cells. After 12 h of incubation, the cultures were examined for HIV-1 cytopathicity (CPE) and the percentage CPE of control for each treatment was recorded. After 5 days of incubation, the MTT test was performed and the EC_{50} and EC_{90} concentrations determined. Each experiment was done twice and the averages of two determinations were recorded.

2.6. HIV reverse transcriptase (RT) assay

The effects of plant extracts on RT activity in vitro were evaluated with recombinant HIV-1 enzyme (Sekagaku Co., Tokyo, Japan) as described previously (Nakashima et al., 1992) except that the reaction mixture contained 5 μl of test plant extract or distilled water. The assays were carried out in triplicate.

2.7. Polymerase chain reaction studies

The effect of plant extracts on HIV proviral DNA copying (HIV proviral DNA-dependent synthesis by taq polymerase) was studied. DNA from HIV-1[$HTLVIII_B$] infected Molt-4 cells was extracted, amplified, and detected as previously published (Ayisi et al., 1995), except that amplification was done in the presence or absence of plant extracts.

3. Results

3.1. Comparative inhibitory effects of plant extracts and AZT against cytopathic effects of HIV-1 and HIV-2 acute infections in Molt-4 cells

Table 1 shows AZT to be extremely effective against HIV-1 strain $HTLVIII_B$ when treatment was started shortly after initiation of infection. Despite the fact that the plant extracts were not pure compounds, the antiviral indices of GHX-2L and GHX-2S were quite good. GHX-6L, GHX-26F, and GHX-27L had moderate anti-HIV indices and GHX-7L was mildly effective. Table 2A shows the effects of plant extracts and AZT tested against HIV-2 strain GH1, when treatment was started at 40 min after infection. With the exception of GHX-26, all plant extracts, particularly GHX-27L, were effective against HIV-2 strain GH1. When treatment was delayed to 2 h post-infection (Table 2B), the plant extracts (except for GHX-7L), were still able to achieve EC_{90} values that were not toxic to Molt-4 clone 8 cells. AZT was not able to achieve 90% inhibition of HIV-2 cytopathicity when treatment was delayed till 2 h post-infection.

3.2. Effects of multiplicity of infection on activities of plant extracts and AZT against HIV-1

Fig. 1 shows the effects of two plant extracts and AZT on HIV-1 strain GH3 at four different multiplicities of infection when treatment was delayed for 2 h. GHX-2L and GHX-6L had similar high to moderate concentration-dependent effects at the four multiplicities of infection. Both plant extracts were able to attain 90% cell protection even at the highest multiplicity of infection (MOI) of 0.114. AZT was very effective except when the MOI was increased to 0.114, under these conditions, 90% cell protection was never attained. The effectiveness of the plant extracts and AZT were inversely related to the MOI.

Table 1
Effects of aqueous extracts of plants against HIV-1 (strain $HTLVIII_B$) in Molt-4 clone 8 acutely infected at an MOI of 0.00357

Plant extract	EC_{50} (mg/ml)	CC_{50} (mg/ml)	AI
GHX-2L	0.01	1.1	110.0
GHX-6L	0.03	1.3	43.3
GHX-7L	0.70	>1.4[a]	≫2.0
GHX-26S	0.02	>1.8[a]	>90.0
GHX-26F	0.01	0.18	18.0
GHX-26S+F	0.01	0.71	71.0
GHX-27L	0.014	0.45	32.0
AZT	<0.000002	0.02	10000.0

Drug treatment was started at 40 min after virus infection. EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; AI, antiviral index defined as CC_{50}/EC_{50}.
[a] Highest concentration tested.

Table 2
Effects of aqueous extracts of plants against HIV-2 (strain GH1) acute infection in Molt-4 clone 8 cells

	Plant extract	EC_{50} (mg/ml)	EC_{90} (mg/ml)	AI
A	GHX-2L	0.075	0.21	14.7
	GHX-2R	0.065	0.15	15.4
	GHX-6L	<0.005[a]	0.17	>260.0
	GHX-7L	0.110	0.40	12.7
	GHX-26S	NE		
	GHX-26F	NE		
	GHX-27L	<0.005[a]	0.02	>90.0
	AZT	<0.0000003[a]	0.0003	>66667.0
B	GHX-2L	0.13	0.32	
	GHX-2R	0.08	0.27	
	GHX-6L	0.025	0.40	
	GHX-7L	0.12	1.25	
	GHX-27L	0.008	0.03	
	AZT	0.000001	NE	

MOI was 0.018, and treatment was started after 40 min (A) or 2 h (B) of virus infection. NE, not effective; AI, antiviral index defined as CC_{50}/EC_{50}.
[a] Lowest concentration tested.

3.3. Comparative effects of plant extracts on the replication of uninfected and chronically infected Molt-4 cells

Fig. 2 shows the effect of plant extract GHX-27L on uninfected and chronically infected Molt-4 cells after treatment for 2 and 4 days. Whereas GHX-27L was toxic to uninfected cells at 0.28 mg/ml, as little as 0.035 mg/ml of this extract was significantly toxic to chronically infected cells. In fact, GHX-27L significantly reduced cell number below pretreatment levels indicating that it did not just stop or inhibit cell replication but in fact it killed the cells. Similar experiments showed that GHX-2L (up to 0.35 mg/ml) and GHX-6L (up to 0.64 mg/ml) did not affect Molt-4 and Molt-4/HIV growth, and that GHX-26F was significantly toxic to both uninfected and chronically infected cells at 0.16 mg/ml.

3.4. Effects of AZT and plant extracts on Molt-4–Molt-4/HIV cocultures

Fig. 3 shows the effects of AZT and plant extracts on cytopathic effects (CPE) observed in cocultures of Molt-4 with Molt-4/HIV after 12 h of incubation. Supernatant taken from 12-h-old untreated cultures did not contain enough virus to produce CPE in fresh Molt-4 cultures within 12 h. AZT had no effect on the CPE produced in the cocultures of Molt-4 with Molt-4/HIV at 12 h of incubation. All the plant extracts tested, on the other hand, showed concentration-dependent inhibition effects. Table 3 shows the results of tetrazolium-based colorimetric assay done after 5 days of incubation. Unlike the plant extracts, AZT was not able to achieve 90% cell protection.

3.5. Effects of plant extracts on HIV-1 reverse transcriptase activity

Fig. 4 shows the anti-HIV-1 RT activities of four plant extracts tested. All plant extracts showed concentration-

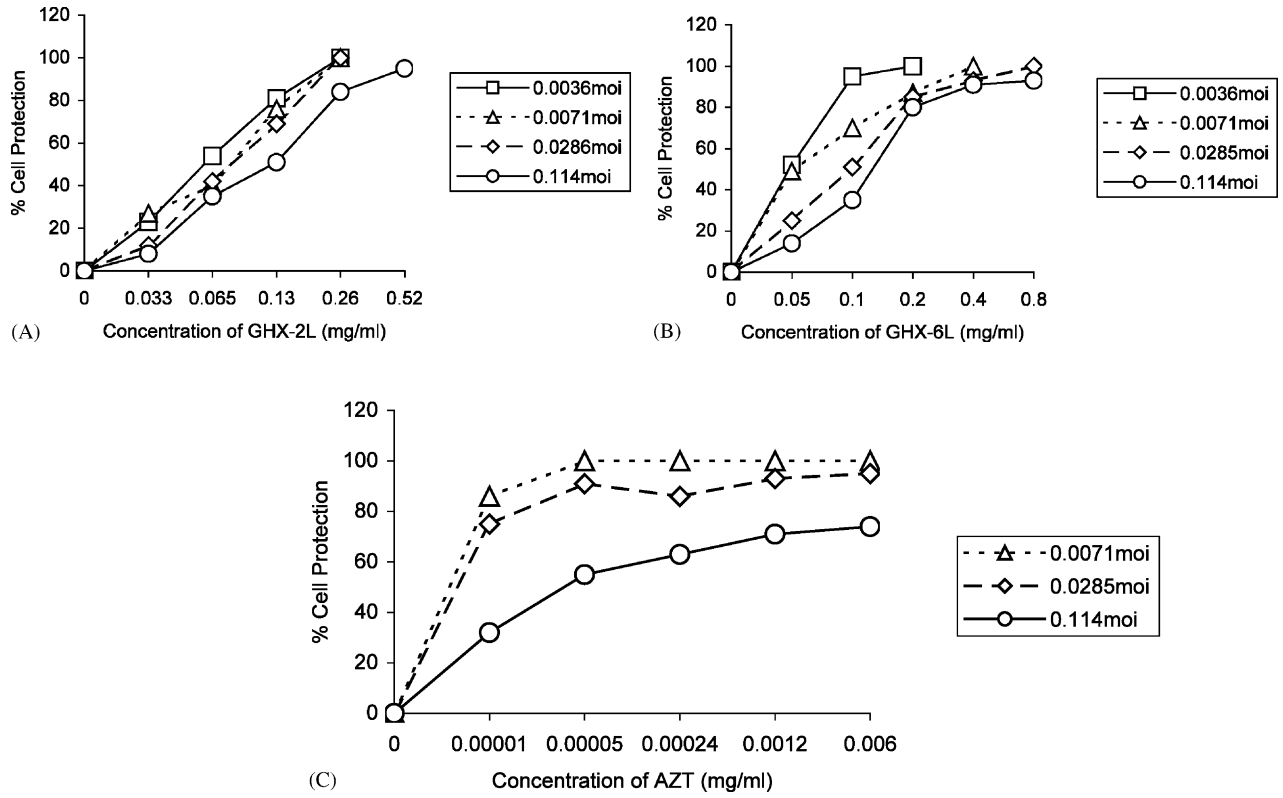

Fig. 1. Effects of MOI on anti-HIV-1 (strain GH3) activities of GHX-2L (A), GHX-6L (B), and AZT (C) in M8166 cells. Treatment was started at post-infection.

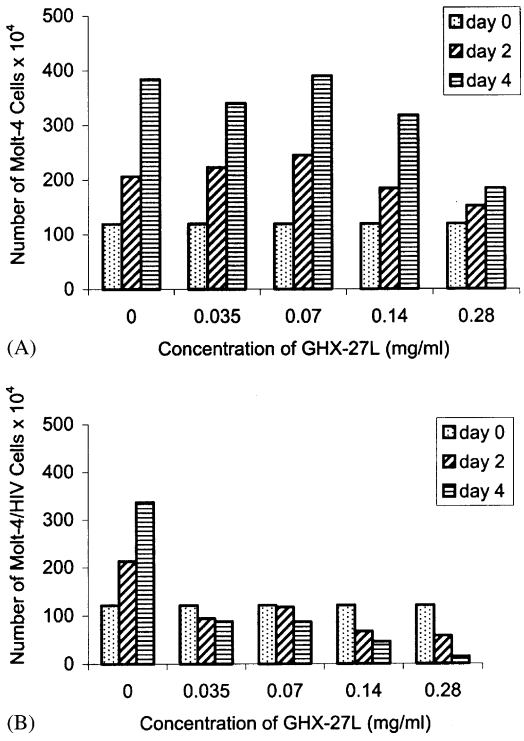

Fig. 2. Effects of plant extract GHX-27L on Molt-4 (A) and Molt-4/HIV (B) cell growth.

Table 3
Effects of AZT and plant extracts GHX-2L, GHX-6L, GHX-26F, and GHX-27L on cocultures of Molt-4 with Molt-4/HIV after 5 days of incubation

Plant extract or drug	EC_{50} (mg/ml)	EC_{90} (mg/ml)
GHX-2L	0.059	0.340
GHX-6L	0.018	0.130
GHX-26F	0.035	0.126
GHX-27L	0.013	0.075
AZT	0.001	NE[a]

The highest cell protection by AZT was 55% at a concentration of 0.005 mg/ml.

[a] NE, not effective.

dependent reductions in RT activity. GHX-2L, GHX-6L, and GHX-26F caused 90% reduction in HIV-1 RT activity at concentrations between 0.013 and 0.020 mg/ml. On the other hand, GHX-27L was not able to cause 90% reduction in RT activity even at the highest concentration of 0.133 mg/ml tested.

3.6. Effects of plant extracts on HIV-1 proviral DNA copying

GHX-2L and GHX-6L were able to stop in vitro HIV-1 proviral DNA copying by taq polymerase at concentrations of 0.011 and 0.015 mg/ml, respectively. A faint but clear

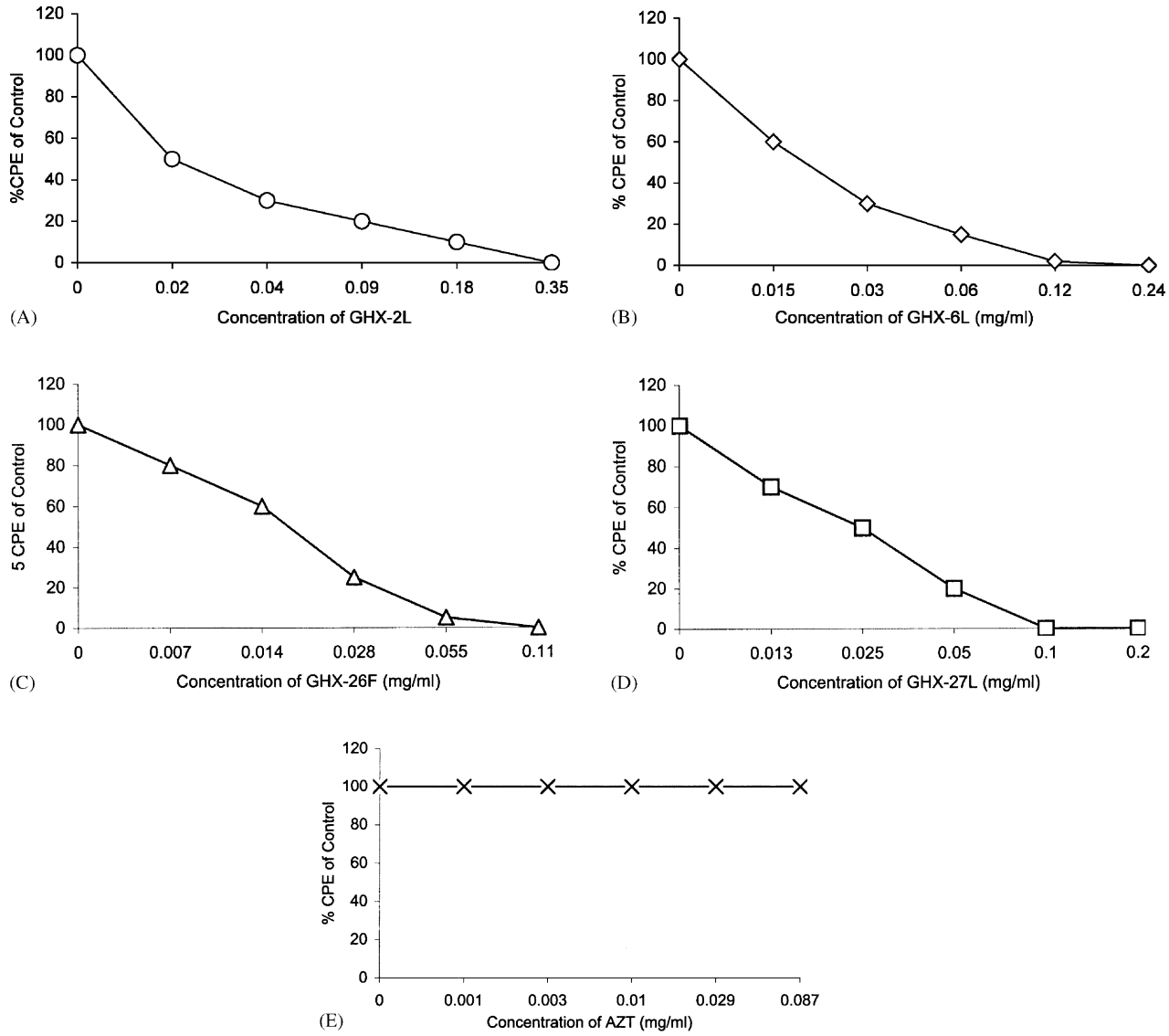

Fig. 3. Effects of GHX-2L (A), GHX-6L (B), GHX-26F (C), GHX-27L (D), and AZT (E) on the cytopathicity in Molt-4 cocultures with Molt-4/HIV after 12 h incubation.

HIV-1 DNA copy band was present for GHX-26F and GHX-27L at 0.016 and 0.058 mg/ml, respectively. Plant extracts GHX-7L and GHX-26S had no effect on HIV-1 DNA-dependent DNA synthesis.

4. Discussion

One of the modes of infection by HIV is acute cytolytic infection. The fact that all five plant extracts had noncytotoxic activities against acute HIV infection indicates that these extracts are acting as true antivirals. This fact is emphasized by the demonstration of MOI-dependent activities, exemplified by GHX-2L and GHX-6L. Virus production in HIV-infected patients is usually high (Pantaleo et al., 1993; Embretson et al., 1993; Wei et al., 1995; Ho et al., 1995), resulting in high in vivo MOI. At low MOI, AZT and plant extracts GHX-2L and GHX-6L were able to achieve 90% inhibition of HIV-1 even when treatment was delayed for 2 h. AZT, unlike the plant extracts, was not able to achieve 90% inhibition of HIV-1 at high MOI under similar conditions.

Despite the predominance of HIV-1 worldwide, a low incidence of HIV-2 and a moderate incidence of dual infection exists in West Africa (Hishida et al., 1994; Ayisi et al., 1995). All plants tested, except for GHX-26, were effective against HIV-2 strain GH1 demonstrating the potential usefulness of these plants in the area where they were collected. At 40 min post-infection, it is expected that adsorption and penetration would have been completed with very little DNA synthesis if any. The ineffectiveness of AZT in achieving considerable viral inhibition against HIV-2 when treatment was started at 2 h post-infection is not surprising since this

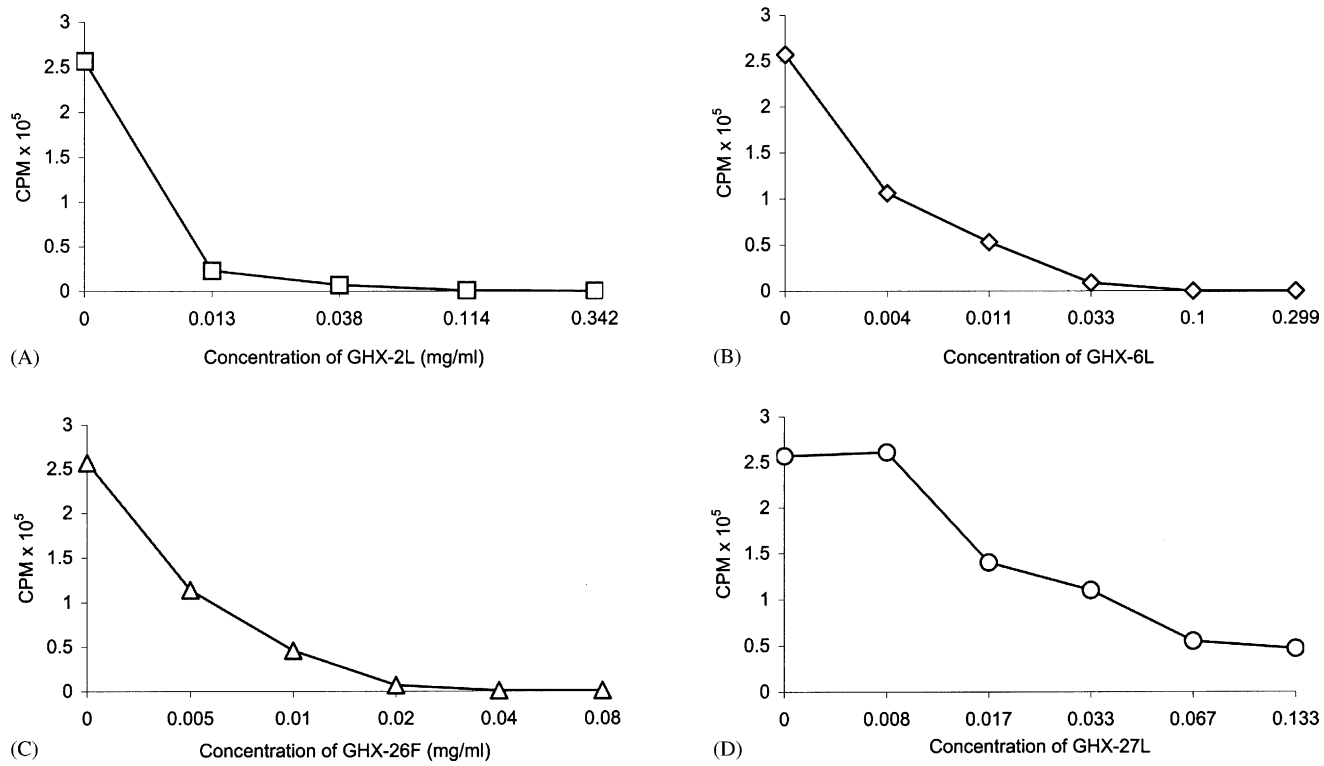

Fig. 4. Effects of GHX-2L (A); GHX-6L (B); GHX-26F (C); and GHX-27L (D) on HIV RT activity.

drug acts only on the early event of RT activity. AZT was, however, very effective against HIV-1 at a similar, low MOI as used for HIV-2. This difference in activity may be due to differences in the rates of HIV-1 and HIV-2 proviral DNA synthesis and integration. This difference in activity is obviated when high MOI of HIV-1 is used. The plant extracts, however, achieved considerable HIV inhibition irrespective of the MOI used and may be active against late viral events.

Several stages of HIV replication serve as targets for drugs (Tuazon and Labriola, 1987; Wong-Staal, 1990). Early events in HIV infection are critical targets because of the role of the RT enzyme, which is unique to this virus and a few others and not found in uninfected cells. This unique enzyme characterizes a two-step process of proviral DNA synthesis (Coffin, 1990). Unintegrated proviral DNA is metabolically active and plays a critical role in the cytopathicity of HIV (Stevenson et al., 1990). Thus, drugs that inhibit RT activity should be beneficial against acute HIV infections. AZT is a known potent inhibitor of both steps of RT activity (Wong-Staal, 1990). GHX-2L and GHX-6L showed consistent high activities against HIV-1 RT in the synthesis of DNA from RNA template (first step) and also inhibited proviral DNA copying by taq polymerase (proviral DNA-dependent DNA synthesis) and thus the two steps in early HIV replication may serve as possible targets for these extracts. GHX-26F while being very effective against RNA-dependent DNA synthesis, was less effective against proviral DNA-dependent DNA synthesis and may have its major activity at RT at the first step. Despite the concentration-dependent anti-RT activities for the tested extracts, this enzyme may not be the primary target for their anti-HIV activities since inhibition of viral cytopathicity was achieved even when treatment was started after proviral DNA synthesis.

When uninfected Molt-4 cells are cocultured with chronically HIV-1-infected Molt-4 cells (Molt-4/HIV), the first sign of HIV cytopathicity was seen within 8–12 h. This is probably due to fusion of uninfected with infected cells. At this stage, not enough virus particles could have been produced by the chronically infected cells to cause such clear cytopathicity within such a short time. In fact, acute HIV-1 infection in Molt-4 cells will take about 72 h for the first clear cytopathicity to be discernible. Thus, the cytopathicity observed within 8–12 h is likely not due to HIV-1 acute infection. This point is further strengthened by the fact that AZT had no effect on this early cytopathicity. The inhibition of this early cytopathicity in uninfected–infected coculture indicates yet another mode of anti-HIV action by the plant extracts. A unique effect on chronically infected cells was observed for plant extract GHX-27L. This extract was selectively toxic to chronically infected cells and thus provides evidence of the possibility of developing drugs with the ability to shut off the virus producing factories by being selectively toxic to chronically infected cells. The fact that GHX-27L significantly reduced chronically infected cell number below pretreatment levels indicates that this extract did not just stop or inhibit cell replication but that in fact it killed the cells. The mechanism underlying the selective

killing of chronically infected cells is unknown but may be due to possible differential uptake of the extract into uninfected and chronically infected cells. This hypothesis will be investigated in future experiments.

Even though the anti-HIV components of the plant extracts have not been studied, phytochemistry work by others shed light on the nature of possible active ingredients for some of them. Eugenol (Nakamura et al., 1999; de Vasconcelos Silva et al., 1999) and thymol (Rodriguez et al., 1997), two phenolic compounds, were found to be antibacterial components of *O. gratissimum*. Ebi (2001) found fractionation components of *A. cordifolia* containing phenolics and terpenoids to have significant antibacterial activities. Even though no phytochemistry has been reported for *F. polita*, other *Ficus* species have been shown to contain various potential antiviral compounds. *Ficus pumila* contains triterpenoids (Kitajima et al., 1998), acetylated triterpenoids (Kitajima et al., 1999), and sesquiterpenoid glucosides (Kitajima et al., 2000). Furanocoumarins, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes have also been isolated from *Ficus carica* (Gibenau et al., 1997). Baumgartner et al. (1990), Khan et al. (1993) isolated alkaloids from *Ficus septica* and *Ficus pachythachis*, respectively. Likewise, even though no phytochemistry has been done for *E. drupifera*, other species of *Euphorbia* have been shown to contain potential anti-HIV compounds. Uemura and Hirata (1971, 1972, 1974) discovered new diterpenoids from *Euphorbia jolikini* (1972) and *Euphorbia kansui Liou* (1974), 13-oxyingenol from *E. Kansui Liou* (1974), and two new alkaloids from *Euphorbia millii* (1971). Cycloeuphordenol, a new triterpene was isolated from *Euphorbia tirucalli* (Khan et al., 1988). Lectins (Lynn and Clevette, 1986) and tannins (Yoshida et al., 1994) have also been isolated from *Euphorbia* species. Ito et al. (1998) isolated the first lactone carbazole alkaloids from a natural source, *C. anisata*. Some phenolics may inhibit HIV adsorption and integration, some terpenoids may inhibit HIV adsorption, virus–cell fusion, and reverse transcription, some alkaloids may inhibit HIV adsorption, reverse transcription and glycosylation, and some coumarins may inhibit HIV adsorption, reverse transcription, integration, protease and assembly/release, some lectins may inhibit HIV–cell fusion, and some tannins may inhibit HIV adsorption and reverse transcription (Vlietinck et al., 1998; Matthee et al., 1999).

In this paper, we showed that aqueous extracts of *O. gratissimum* (GHX-2), *F. polita* (GHX-6), *C. anisata* (GHX-7), *A. cordifolia* (GHX-26), and *E. drupifera* (GHX-27) are effective inhibitors of HIV-1 and HIV-2 replication. The plant extracts unlike AZT were able to achieve significant inhibition of viral cytopathicity even at high MOI when treatment was delayed for 2 h. Early fusion of chronically HIV-infected cells with uninfected cells has been shown to be unaffected by AZT but to be inhibited by GHX-2, GHX-6, GHX-26, and GHX-27. Significantly, an extract of *E. drupifera* has been identified to be selectively toxic to chronically infected cells at concentrations that are not toxic to uninfected cells. In order to further delineate their therapeutic potentials in clinical HIV infections/AIDS, the plant extracts will have to be tested against other strains of HIV-1 and -2, including those that are resistant to AZT.

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