

# Argentine Plant Extracts Active Against Polymerase and Ribonuclease H Activities of HIV-1 Reverse Transcriptase

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**Lipophilic and hydrophilic extracts of four Argentine plants (*Gamochaeta simplicaulis* Cabr. 1, *Achyrocline flaccida* Wein. D. C. 2, *Eupatorium buniifolium* H. et A. 3, and *Phyllanthus sellowianus* Muell. Arg. 4) were examined *in vitro* for their ability to inhibit the polymerase and ribonuclease H (RNase H) activities of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) (wild and Y181C mutant types). The active extracts were also examined as inhibitors of viral replication in HLT4LacZ-1<sub>III</sub>B cell cultures, evaluating their cytotoxicity in parallel. Infusions 2I and 4I, among the crude extracts, showed the highest activity. These extracts were refractionated into four fractions; 2I<sub>4</sub> and 4I<sub>4</sub> were active as inhibitors of DNA-polymerase (wild and Y181C types) and RNase H activities. These fractions were potent as inhibitors of viral replication and were not cytotoxic. Refractionation of 2I<sub>4</sub> yielded five new fractions, two of which, 2I<sub>4</sub>-4 and 2I<sub>4</sub>-5, showed notable activity. Refractionation of 4I<sub>4</sub> yielded four new fractions; of these, 4I<sub>4</sub>-3 and 4I<sub>4</sub>-4 were active. The marked biological activity found in the infusion of *A. flaccida* and *P. sellowianus* makes them sufficiently attractive to be considered in the combined chemotherapy of the disease. Copyright © 1999 John Wiley & Sons, Ltd.**

**Keywords:** *Gamochaeta simplicaulis* Cabr.; *Achyrocline flaccida* Wein. D. C.; *Eupatorium buniifolium* H. et A.; *Phyllanthus sellowianus* Muell. Arg.; HIV-1 RT; RNase H.

## INTRODUCTION

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is a multifunctional enzyme exhibiting both RNA and DNA dependent DNA-polymerase activities as well as an inherent ribonuclease H (RNase H) activity. The enzyme is considered an ideal target for the chemotherapeutic intervention of AIDS and, in fact, compounds, such as 3'-azido-2',3'-dideoxythymidine, that inhibit its function are used in the treatment of the disease. However, their clinical use is limited due to their toxicity and the emergence of resistant viral strains.

Extracts from four selected Argentine medicinal species have been examined *in vitro* for their ability to inhibit the HIV-1 RT DNA-polymerase and RNase activities.

*Gamochaeta simplicaulis* Cabr. 1, common name 'plan cachu', is used in folk medicine against pock, measles and varicella; and it is used externally for lavages of sores and pimples (Zardini, 1984). Infusions of *Achyrocline flaccida* Wein. D. C. 2 (common name: 'marcela', 'marcela macho') are used as an antispasmodic, febrifuge, stimulant, emenagogue, excitant and antihelmintic

(Zardini, 1984). It is also used in external preparations for healing cuts, lacerations and ulcerations of the skin. Extracts of this plant have been reported to have antimicrobial activity (Gutkind *et al.*, 1984). Investigations of the antiherpetic activity of both species has been previously reported (Cavallaro *et al.*, 1995; García *et al.*, 1995). *Eupatorium buniifolium* H. et A. 3, common name 'romero', 'romerillo', 'romero colorado', is used as a tincture for its hepatoprotective and disinfectant properties (Zardini, 1984). *Phyllanthus sellowianus* Muell. Arg. 4 (Euphorbiaceae), commonly known as 'sarandi blanco', is a shrub native to South America, widely distributed in Paraguay, Northeastern Argentina, Southern Brazil and Uruguay. It is a well-known Argentine medicinal plant and it is quoted in the Argentine Pharmacopeia (1978) as a hypoglycaemic, diuretic, laxative and antiseptic in folk medicine (Hieronymus, 1882; Sorarú and Bandoni, 1978). This species was selected based on the activity reported for other species of this genus against hepatitis B virus (Unander *et al.*, 1995).

## MATERIAL AND METHODS

**Plant Material.** *Gamochaeta simplicaulis* aerial parts were collected in Buenos Aires Province, November 1991; *Achyrocline flaccida*, aerial part in Entre Ríos

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**Table 1. Activity of crude extracts**

Extract	HIV-1 DNA-polymerase			HIV-1 ribonuclease H Antiinfective activity			
	Wild type	Y181C					
	% Inh. <sup>a</sup> (100 µg/mL)	IC <sub>50</sub> <sup>b</sup> (µg/mL)	% Inh. <sup>a</sup> (100 µg/mL)	IC <sub>50</sub> <sup>b</sup> (µg/mL)	% Inh. <sup>a</sup> (100 µg/mL)	IC <sub>50</sub> <sup>b</sup> (µg/mL)	HLT4LacZ-1 <sub>III</sub> B IC <sub>50</sub> <sup>a</sup> (µg/mL)
1I	99 ± 0.14	36.0 ± 5	90 ± 12.0	nd <sup>c</sup>	49 ± 6.4	-	-
1M	92 ± 3.0	20.0 ± 1	90 ± 1.1	nd	61 ± 1.9	nd	>2.0
1C	15 ± 6.2	-	5 ± 3.5	-	26 ± 5.4	-	-
2I	93 ± 9.5	3.9 ± 0.1	82 ± 18.0	nd	97 ± 1.6	10.0 ± 2.5	1.1
2I <sub>1</sub>	11 ± 9.9	-	19 ± 3.1	-	26 ± 10.7	-	-
2I <sub>2</sub>	9 ± 14.3	-	18 ± 1.2	-	52 ± 9.8	-	-
2I <sub>3</sub>	17 ± 9.3	-	16 ± 5.0	-	53 ± 12.0	-	-
2I <sub>4</sub>	98 ± 1.9	1.0 ± 0.1	92 ± 0.3	1.8 ± 0.6	96 ± 0.3	3.9 ± 0.9	0.25
2I <sub>4</sub> -1	4 ± 4.7	-	2 ± 7.8	-	nd	nd	-
2I <sub>4</sub> -2	25 ± 1.1	-	17 ± 4.5	-	76 ± 3.1	nd	-
2I <sub>4</sub> -3	85 ± 4.9	43.0 ± 4.0	85 ± 5.5	26.0 ± 2.5	63 ± 2.7	nd	-
2I <sub>4</sub> -4	98 ± 1.1	17.0 ± 5.0	100 ± 0.9	1.2 ± 0.5	72 ± 3.0	12.8 ± 6.8	nd
2I <sub>4</sub> -5	99 ± 1.1	12.0 ± 0.8	100 ± 0.9	7.3 ± 0.2	100 ± 0.9	5.4 ± 1.8	1.4
2M	36 ± 1.6	-	31 ± 14.0	-	40 ± 3.1	-	-
2C	17 ± 3.2	-	8 ± 2.5	-	31 ± 3.2	-	-
3I	96 ± 0.2	16.0 ± 1.0	90 ± 5.9	nd	100 ± 0.8	3.6 ± 2.8	5.0
3M	35 ± 9.9	-	21 ± 3.8	-	37 ± 0.4	-	-
3C	4 ± 5.6	-	1 ± 1.4	-	19 ± 4.4	-	-
4I	99 ± 0.1	4.1 ± 0.5	96 ± 3.8	nd	94 ± 6.2	17.0 ± 3.2	1.6
4I <sub>1</sub>	11 ± 11.0	-	17 ± 9.0	-	27 ± 1.0	-	-
4I <sub>2</sub>	8 ± 4.2	-	4 ± 6	-	32 ± 5.7	-	-
4I <sub>3</sub>	57 ± 9.6	-	50 ± 6.8	-	46 ± 14.0	-	-
4I <sub>4</sub>	100 ± 0.9	2.4 ± 0.8	91 ± 0.3	3.7 ± 0.6	96 ± 1.7	5.9 ± 1.4	3.0
4I <sub>4</sub> -1	10 ± 5.6	-	6 ± 0.6	-	31 ± 4.5	-	-
4I <sub>4</sub> -2	21 ± 0.6	-	11 ± 3.2	-	42 ± 8.3	-	-
4I <sub>4</sub> -3	100 ± 0.9	2.4 ± 0.2	100 ± 0.3	1.7 ± 0.2	95 ± 2.4	3.0 ± 0.4	1.2
4I <sub>4</sub> -4	100 ± 0.7	1.7 ± 0.1	100 ± 0.3	1.7 ± 0.6	95 ± 0.1	2.6 ± 0.5	1.8
4M	77 ± 1.4	52.0 ± 2.0	75 ± 1.4	nd	62 ± 0.7	nd	-
4C	22 ± 1.9	-	14 ± 1.3	-	33 ± 7.0	-	-
AZT	-	-	-	-	-	-	1.21 <sup>e</sup>
U-	-	0.21 ± 0.03 <sup>d</sup>	-	4.5 ± 0.2 <sup>d</sup>	nd	-	14.0 <sup>e</sup>
90152s	-	-	-	-	-	-	-
L-	-	0.19 ± 0.05 <sup>d</sup>	-	28.3 ± 4.3 <sup>d</sup>	nd	-	20.0 <sup>e</sup>
697661	-	-	-	-	-	-	-

<sup>a</sup> Mean ± SEM (n = 3–5)<sup>b</sup> Concentration-activity curves were carried out with four or more concentrations of test extracts; IC<sub>50</sub> values were calculated from log curve.<sup>c</sup> nd, no data;<sup>d</sup> µM;<sup>e</sup> nM.

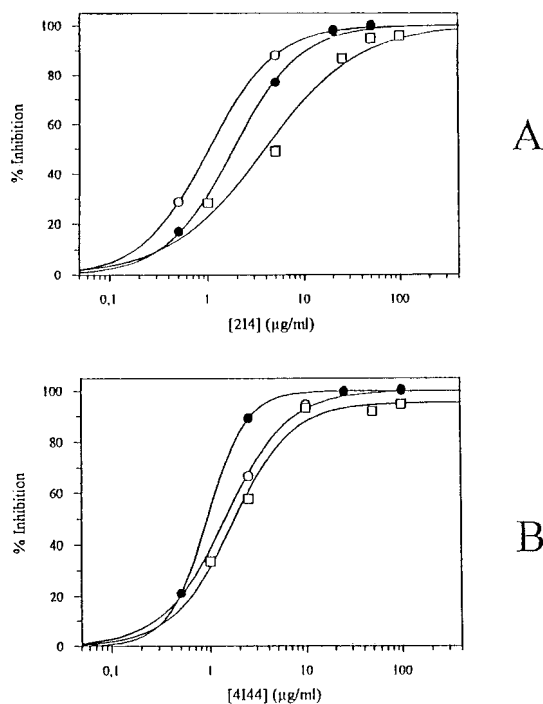
Province, February 1991; *Eupatorium buniifolium* aerial parts and *Phyllanthus sellowianus*, stem bark were collected in Entre Ríos Province, February, 1988. Voucher samples are deposited in the Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

**Preparation of extracts.** Hot water infusions (I) were prepared according to the Argentine Pharmacopoeia VI and lyophilized. Dichloromethane (C) and methanol extracts (M) were also prepared and taken to dryness together under reduced pressure. The marc from the CH<sub>2</sub>Cl<sub>2</sub> extraction was extracted twice with methanol (100 mL each), following the same procedure as for the dichloromethane extract.

The infusion of *P. sellowianus* (4I) was extracted successively with CH<sub>2</sub>Cl<sub>2</sub>, Et<sub>2</sub>O and AcEt. The aqueous remaining fraction (4I<sub>4</sub>) was chromatographed on a Sephadex LH20 column (15 × 2 cm). Four fractions were eluted using MeOH, MeOH–H<sub>2</sub>O 7:3, MeOH–H<sub>2</sub>O 5:5 and H<sub>2</sub>O. The infusion of *Achyrocline flaccida* (2I) was

extracted in a liquid/liquid extractor successively with CH<sub>2</sub>Cl<sub>2</sub>, Et<sub>2</sub>O and AcEt. The aqueous remaining fraction (2I<sub>4</sub>) was lyophilized, redissolved in water and centrifuged. The supernatant was precipitated by adding a BaCl<sub>2</sub> saturated solution. The precipitate was centrifuged and the pellet discarded. The supernatant was chromatographed on a Sephadex LH20 column (15 × 2 cm) eluted with MeOH, MeOH–H<sub>2</sub>O 5:5 and H<sub>2</sub>O, affording five fractions.

**Enzyme assays.** Wild type and mutated (Y181C) recombinant reverse transcriptases (p66/p66) were purified by immobilized metal affinity chromatography (Imac) as described (Sharma *et al.*, 1991). RNA directed DNA polymerase activity was measured as previously described (Font *et al.*, 1995). RNase H activity was measured as described by Zhan *et al.*, (1994). Infusion extracts were solubilized in water whereas methanol and dichloromethane extracts were solubilized in DMSO, diluted in water and assayed, maintaining the final concentration of DMSO at 0.5% (v/v).



**Figure 1.** Dose-response curves of inhibition of the catalytic functions of HIV-1 RT by (A) 2I<sub>4</sub> and (B) 4I<sub>4</sub>-4 fractions. (○) wild type RNA-dependent DNA polymerase activity; (□) wild type RNAse H activity; (●) Y181C RNA-dependent DNA polymerase activity.

**Cell cultures.** HIV-1<sub>IIIB</sub> chronically infected Molt-3 cells (Molt-3/HIV-1<sub>IIIB</sub>), kindly provided by F. Barin and B. Janvier from Université François Rabelais (Tours, France) and HLT4LacZ-1 cells, kindly provided by S. Saragasti from Hôpital Cochin (Paris, France), were cultured according to previously described methods (Font *et al.*, 1995).

**Syncytia formation assay.** According to previously described methods (Font *et al.*, 1995) in HLT4LacZ-1 cells.

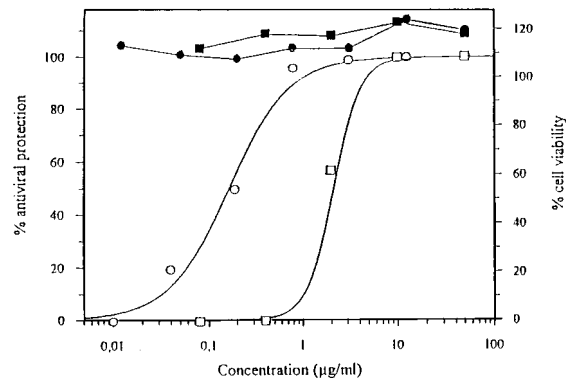
**Cell toxicity.** According to previously described methods, in HLT4LacZ-1 cells (Aggarwal *et al.*, 1985; Font *et al.*, 1995).

## RESULTS

Among the crude extracts the highest activity was observed in the hydrophilic extracts, especially in the infusions 2I and 4I. Among the methanol extracts, only 1M and 2M presented slight activity. All the lipophilic extracts (CH<sub>2</sub>Cl<sub>2</sub>) were inactive (Table 1).

Extracts 2I and 4I were refractionated into four fractions. The fractions 2I<sub>4</sub> and 4I<sub>4</sub> showed activity in the inhibition of the DNA-polymerase activity of wild and mutant types as well as in the inhibition of the ribonuclease activity (Fig. 1a); in addition, these fractions were potent as inhibitors of viral replication and were non cytotoxic (Fig. 2).

Refractionation of 2I<sub>4</sub> yielded five new fractions, two of which (2I<sub>4</sub>-4 and 2I<sub>4</sub>-5) showed activity. Refractionation of 4I<sub>4</sub> yielded four new fractions; of these four, 4I<sub>4</sub>-3



**Figure 2.** Effect of selected fractions on virus replication and cytotoxicity on HLT4LacZ-1 cells. (○) 2I<sub>4</sub> antiviral activity; (□) 4I<sub>4</sub>-4 antiviral activity; (●) 2I<sub>4</sub> cytotoxicity; (■) 4I<sub>4</sub>-4 cytotoxicity.

and 4I<sub>4</sub>-4 were active (Fig. 1b and Fig. 2). U-90152s, L-697661 and AZT were used as reference patterns.

## DISCUSSION

Many of the RT inhibitors obtained from synthetic or natural sources have been reported to affect the DNA polymerase activity of RT, but there are only a limited number of compounds that have been found to inhibit both polymerase and RNase H activities. It has been shown that the monophosphorylated form of AZT can inhibit RNase, but at much higher concentrations than that needed to inhibit the polymerase activity (Tan *et al.*, 1991). On the other hand, nevirapine, a nonnucleoside RT inhibitor, has been found to alter the cleavage specificity of RNase but it was unable to inhibit this activity (Palaniappan *et al.*, 1995). It has been reported that small naphthalenesulfonic acid derivatives were able to inhibit both activities, but their potency as RNase H inhibitors was over tenfold to 100-fold lower compared with the polymerase activity (Mohan *et al.*, 1994). These derivatives showed poor activity when tested in the whole virus assay, probably due to a lack of cellular entry.

A number of substances have been isolated from natural sources that inhibit the RNase H of RT. Of these, suramin and illimaquinone have been shown to specifically inhibit this activity, whereas 3,5,8-trihydroxy-4-quinolone, avarol and avarone derivatives, obtained from sponges from the Red Sea, preferentially inhibited the polymerase activity (Mitsuya *et al.*, 1984; Loya *et al.*, 1990, 1994). However, these compounds turned out to be either insufficiently potent or too toxic to be considered as potential anti-HIV drugs.

In this paper we describe the inhibition of DNA polymerase and RNase H activity of HIV-1 RT mediated by extracts obtained from four Argentine medicinal plants. Infusions of *Achyrocline flaccida* and *Phyllanthus sellowianus* were the most active. More interestingly, they were active when tested on a mutated enzyme, in which a cysteine substitutes a tyrosine in position 181 (Y181C); this form of mutation confers cross resistance to the majority of the nonnucleoside inhibitors. In addition, these aforementioned extracts are also capable of inhibiting the RNase H activity, they are active in

antiviral assays and do not show cytotoxicity in the range of concentrations tested. The correlation between their potency and the antiviral assay reinforces the idea that the mechanism of action of these extracts is due to RT inhibition. The marked biological activity found in the infusions of *A. flaccida* and *P. sellowianus* makes

them sufficiently attractive to be considered in the combined chemotherapy of the disease. Bioguided fractionation of the active extracts is being carried on and studies to characterize the active principles and to determine the mechanism of these inhibitors are currently in progress.

## REFERENCES

- Aggarwal, B. B., Kohr, W. S., Hass, P. E. et al. (1985). Human necrosis factor. Production, purification and characterization. *J. Biol. Chem.* **260**, 2345–2354.
- Cavallaro, L., García, G., Broussalis, A. et al. (1995). Antiherpetic *in vitro* activity of *Gamochaeta simplicicaulis* extract. *Phytother. Res.* **9**, 176–179.
- Farmacopea Nacional de Argentina (1978) 6th edn p. 810, Buenos Aires.
- Font, M., Monge, A., Cuartero, A. et al. (1995). Indole and pyridazinol[4,5-b]indoles as nonnucleoside analog inhibitors of HIV-1 reverse transcriptase. *Eur. J. Med. Chem.* **30**, 963–971.
- García, G., Cavallaro, L., Broussalis, A. et al. (1995). Antiviral activity of *Achyrocline flaccida* Wein DC aqueous extract. *Phytother. Res.* **9**, 251–254.
- Gutkind, G., Norbedo, C., Mollerach, M., Ferraro, G., Coussio, J. and De Torres, R. (1984). Antimicrobial activity of *Achyrocline flaccida*. *J. Ethnopharmacol.* **10**, 319–321.
- Hieronymus, J. (1882). *Plantae Diaforicae, Florae Argentinae*, Boletín de la Academia Nac. de Ciencias, Totno IV, p. 446 Buenos Aires.
- Loya, S., Rudi, A., Tal, R., Kashman, Y., Loya, Y., Hizi, A. (1994). 3,5,8-Trihydroxy-4-quinolone, a novel natural inhibitor of the reverse transcriptase of human immunodeficiency viruses type 1 and type 2. *Archiv. Biochem. Biophys.* **309**, 315–322.
- Loya, S., Tal, R., Kashman, Y. and Hizi, A. (1990). Illimaquinone, a selective inhibitor of the RNase H activity of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **34**, 2009–2012.
- Mitsuya, H., Popovic, M., Yarchoan, R., Matsushita, S., Gallo, R. C. and Broder, S. (1984). Suramin protection of T-cells *in vitro* against infectivity and cytopathic effect of HLTV-III. *Science* **226**, 172–174.
- Mohan, P., Loya, S., Avidan, O. et al. (1994). Synthesis of naphthalene sulfonic acid small molecules as selective inhibitors of the DNA polymerase and ribonuclease H activities of HIV-1 reverse transcriptase. *J. Med. Chem.* **37**, 2513–2519.
- Palaniappan, C., Fay, P. J. and Bambara, R. A. (1995). Nevirapine alters the cleavage specificity of ribonuclease H of human immunodeficiency virus 1 reverse transcriptase. *J. Biol. Chem.* **279**, 4861–4869.
- Sharma, S. K., Evans, D. B., Vosters, A. F., McQuade, T. J. and Tarpley, W. G. (1991). Metal affinity chromatography of recombinant HIV-1 reverse transcriptase containing a human renin cleavable metal binding domain. *Biotechnol. Appl. Biochem.* **14**, 69–81.
- Sorará, S. and Bandoni, A. (1978). *Plantas de la Medicina Popular* p. 55. De Albatros SRL, Buenos Aires.
- Tan, C.-K., Civil, R., Mian, A. M., So, A. G. and Downey, K. M. (1991). Inhibition of RNase H activity of HIV reverse transcriptase by azidothymidylate. *Biochemistry* **30**, 4831–4835.
- Unander, D., Webster, G., Blumberg, B. (1995). Usage of a bioassay in *Phyllanthus* (Euphorbiaceae) IV clustering of antiviral uses and other effects. *J. Ethnopharmacol.* **45**, 1–18.
- Zardini, E. (1984). Etnobotánica de compuestas Argentinas con especial referencia a su uso farmacológico. *Acta Farmacéutica Bonaerense* **3**, 77–95.
- Zhan, X., Tan, C. K., Scott, W. A., Mian, A. M., Downey, K. M. and So, A. G. (1994). Characterization of the RNase H activity of HIV-1 reverse transcriptase. *Biochemistry* **33**, 1366–1372.