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SHORT COMMUNICATION

Inhibitory Effect Against Polymerase and Ribonuclease Activities of HIV-reverse Transcriptase of the Aqueous Leaf Extract of *Terminalia triflora*

V. S. Martino,[1]* P. López,[1] J. J. Martinez Irujo,[2] M. Sanromán,[2] M. T. Cuevas,[2] E. Santiago,[2] J. J. Lasarte,[2] M. Font,[3] J. D. Coussio[1] and A. Monge[2]

[1]Cátedra de Farmacognosia, Instituto de Química y Metabolismo del Fármaco (IQUIMEFA-PROSIVAD) (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, República Argentina
[2]Centro de Investigación en Farmacobiología Aplicada (CIFA)
[3]Departamento de Química Orgánica y Farmacéutica de la Facultad de Farmacia, Universidad de Navarra, 31080, Pamplona, España

Dichloromethane, methanol and aqueous extracts from the leaves of *Terminalia triflora* were investigated for their inhibitory effect on polymerase and ribonuclease activities of HIV reverse transcriptase.The most potent activity was found in the aqueous extract, which inhibited both polymerase and ribonuclease activities of the enzyme with an IC_{50} of 1.6 μg/mL and 1.8 μg/mL respectively. The antiinfective activity of the extract was demonstrated in HLT4LacZ-IIIB cell culture with an IC_{50} of 1.0 μg/mL. The extract was submitted to a purification process by extractive and chromatographic methods. The activity remained in the hydrophillic fraction. Tannins present in this active purified fraction, as determined by TLC and HPLC methods, could account for the anti HIV-RT activity found in the aqueous extract.

Keywords: Terminalia triflora; aqueous extract; antiHIV-reverse transcriptase activity.

INTRODUCTION

Reverse transcriptase (RT) is an enzyme that plays an important role in the replication of human immunodeficiency virus (HIV) and is considered a key target in the chemotherapy of the disease. Many compounds derived from plants, such as alkaloids, coumarins, tannins and triterpenes (Vlietinck *et al.*, 1998), that inhibit RT have been identified, but the plant kingdom should be explored further in the search for new leading compounds to develop more effective drugs with fewer side effects.

In the course of a screening project (see Hnatyszyn *et al.*, 1999) looking for new natural sources of anti-HIV drugs, *Terminalia triflora* was included for study. The selection of this species was based on the findings of Planes de Banchero and Souto *et al.* (1969), Rivas *et al.* (1971) and Rivas and Distefano (1973) who had shown that leaf extracts of this plant were active against several RNA viruses such as rabies, foot and mouth disease and influenza.

Terminalia triflora (Griseb.) Lillo (Combretaceae) is a native tree which is common throughout the Northern and Northeastern regions of Argentina (Digilio and Legname, 1966). It is known by the common names of lanza, lanza amarilla, amarillo de río or palo amarillo. There are no records of the use of this plant in folk medicine, but Hieronymus (1882) mentions the use of its bark in the making of posts, furniture, weapons for soldiers and as fuel. Nevertheless, many other exotic species of the genus *Terminalia* have medicinal properties and some antiHIV compounds have been isolated from them (El Mekkawy *et al.*, 1995; Kusumoto *et al.*, 1995).

Previous phytochemical studies of *T. triflora* leaves have led to the characterization of saponins, steroids, phenolic compounds and tannins (Rondina *et al.*, 1970); quercitrin, afzelin, ellagic and gallic acids, methylgallate and two hydrolysable tannins have been isolated and identified from the same species (Martino *et al.*, 1975).

The aim of this investigation was to evaluate an aqueous leaf extract of *T. triflora* against the polymerase and ribonuclease activities of HIV-RT and evaluate its potential as a source of new leading antiHIV compounds.

MATERIALS AND METHODS

Plant material. The leaves of *T. triflora* were collected in Tucumán Province, Departamento Capital, in February 1995. Botanical authentication was carried out by Ing. A. Slanis from the Instituto Miguel Lillo (Tucumán) and a voucher specimen is deposited at the Herbarium (A. Slanis 552).

* Correspondence to: V. S. Martino, Catedra de Farmacognosiá Instituto de Química y Metabolismo del Fármaco (IQUIMEFA-PROSIVAD) (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 1113 Buenos Aires, República Argentina.
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Table 1. Inhibitory effect of *Terminalia triflora* extracts on HIV-RT

Extract/ compound	HIV-1 DNA-polymerase				HIV-1 ribonuclease H		Anti-infective activity
	Wild Type		Y181C				
	% Inh.[a] (100 μg/mL)	IC_{50}[b] (μg/mL)	%Inh[a] (100 μg/mL)	IC_{50}[b] (μg/mL)	% Inh[a] (100 μg/mL)	IC_{50}[b] (μg/mL)	HLT4LacZ-1_{IIIB} IC_{50} (μg/mL)
1I	96 ± 4.7	1.6 ± 0.1	92 ± 6.5	–	94 ± 3.4	1.8 ± 0.12	1.0
$1I_1$	41 ± 4.3	–	56 ± 4.2	–	67.4 ± 4.5	20.5 ± 6.0	–
$1I_2$	87 ± 5.3	5.8 ± 1.4	81 ± 5.3	19.0 ± 6.7	88 ± 3.2	5.0 ± 0.5	2.0
$1I_3$	96 ± 2.2	4.7 ± 0.9	88 ± 2.5	5.6 ± 0.6	97 ± 0.5	1.3 ± 0.22	0.3
$1I_3$-1	55 ± 5.5	91 ± 9.1	36 ± 2.0	–	53 ± 8.0	–	–
$1I_3$-2	99.7 ± 0.8	1.0 ± 0.11	99.5 ± 0.35	1.7 ± 0.2	97 ± 0.9	0.8 ± 0.1	0.2
$1I_3$-3	99.9 ± 0.9	0.9 ± 0.14	99.7 ± 0.6	0.9 ± 0.1	97 ± 0.6	0.7 ± 0.05	0.3
$1I_3$-4	99.9 ± 0.6	1.1 ± 0.24	99.9 ± 0.14	1.2 ± 0.05	95 ± 4.2	1.5 ± 0.1	0.6
M	92 ± 9.7	2.7 ± 0.27	–	–	98 ± 1.5	2.9 ± 1.1	<1.6
DM	42 ± 3.9	–	25 ± 6.4	–	35 ± 6.1	–	–
AZT	–	–	–	–	–	–	1.2[c]
U-901521	–	0.2 ± 0.1[d]	–	4.5 ± 0.17[d]	–	–	14.0[c]
L-697661	–	0.1 ± 0.06[d]	–	28.3 ± 4.2[d]	–	–	20.0[c]

[a] Mean value ± SD.
[b]Mean value ± SD.
Concentration-activity curves were carried out with four or more concentrations of the tested extracts. IC_{50} values were calculated by nonlinear regression as described (Villahermosa *et al.*, 1997), using the commercial available fitting program Grafit (Erithacus software).
[c] nM
[d] μM;
–, not determined. DM, dichloromethane; M, methanol. I, aqueous extract.

Dichloromethane and methanol extracts. 5 g of the dried ground material was extracted at room temperature with three portions of 100 mL of dichloromethane each, for 24 h. The extracts were filtered, taken to dryness under reduced pressure and labelled DM. The plant material was further extracted with methanol following the same procedure as for the dichloromethane extract and labelled M.

Aqueous extract. 10 g of the dried plant material was placed in a stoppered flask and 100 mL of hot water was added. The mixture was left standing for 20 min. The extract was filtered, freeze-dried and labelled 1I.

Enzyme assays. U-901521 and L-697661 used as positive control were kindly provided by Upjohn Co., Kalamanzoo, USA.

Wild type and mutated (Y181C) recombinant reverse transcriptases (p66/p66) were purified by immobilized metal affinity chromatography (IMAC) as described by Sharma *et al.* (1991). RNA directed DNA polymerase activity was measured as previously described by Font *et al.* (1995). Ribonuclease activity was measured as described by Zhan *et al.* (1994). The aqueous extract was dissolved in water while the methanol and dichloromethane extracts were taken up in DMSO, diluted with water and assayed, maintaining the final concentration of DMSO at 0.5% (v/v). Concentration–activity curves were carried out with four concentrations of the tested extracts. IC_{50} values were estimated from the log curve.

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

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

Cell toxicity. Cell toxicity was measured according to

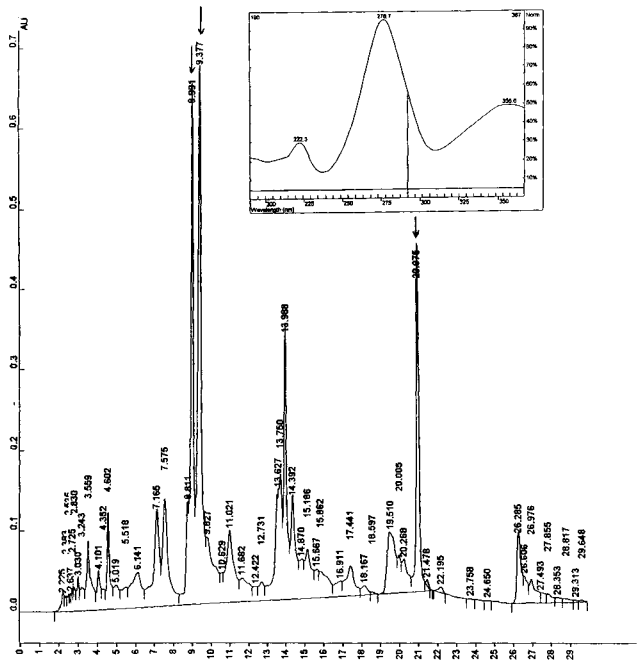

Figure 1. HPLC chromatogram of fraction II_3, UV profile of mayor peaks (tr = 9.0; 9.4; 21.0) is shown at the upper right corner.

previously described methods in in HLT4LacZ-1 cells (Font *et al.*, 1995).

Fractionation of the aqueous extract. The aqueous extract (1I) was partitioned in a liquid/liquid apparatus with diethyl ether ($1I_1$) and ethyl acetate ($1I_2$). The remaining aqueous fraction ($1I_3$) was chromatographed on a Sephadex LH-20 column (15 × 2 cm). Four fractions were eluted using methanol ($1I_3$-1), methanol–water 7:3 ($1I_3$-2); methanol–water ($1I_3$-3) 5:5 and water ($1I_3$-4).

TLC analysis. Cellulose 300MN plates were used with AcOH 6% as developing solvent and spots were detected with UV light (366 nm) and by spraying with $FeCl_3$.

HPLC analysis. The HPLC equipment used was a Varian with a photodiode Polychrom 9065.

A IB-Sil RP18 column (250 × 4.6 mm, 5 μm) was used. Detection was carried out using a Varian photodiode array detector. A gradient was applied using H_2O–AcOH 98:2 as solvent A and MeOH:AcOH 98:2 as solvent B from 90% A to 60% A in 20 min; 60% A to 100% B in 10 min. The flow rate was 1.0 mL/min. The elution profile was monitored at 280 nm.

RESULTS AND DISCUSSION

The inhibitory activity on polymerase and ribonuclease activities of HIV-RT for both aqueous and methanol extracts is shown in Table 1 and was observed to be dose dependent. An interesting inhibition was shown by the aqueous extract (1I). More interestingly, the aqueous extract was active when tested on a mutated enzyme and inhibited ribonuclease activity at almost the same concentration that inhibited polymerase activity. The importance of this finding is that there is only a limited number of compounds capable of inhibiting both activities of the enzyme at the same dose. The monophosphorylated form of AZT, as described by Tan *et al.* (1991) can inhibit Rnase, but at a much higher concentration than that needed for inhibition of polymerase activity. The aqueous extract proved to be a potent inhibitor of virus replication in the HLT4lacZ1 cell culture and was non toxic (data not shown) at the concentration in which 94% of inhibition was observed. This makes the aqueous extract of *T. triflora* an attractive source of bioactive leading compounds.

Bioguided assay fractionation of the active aqueous extract was undertaken in order to isolate its HIV-RT inhibiting principles. Extractive and chromatographic methods led to a purified active fraction ($1I_3$-3), which by TLC and HPLC analysis showed the presence of several major compounds with the characteristic ultraviolet profile of tannin compounds (Fig. 1).

Tannins have been reported to have HIV-RT inhibitory activity (Nonaka *et al.*, 1990; El Mekkawy *et al.*, 1995). The presence of tannins in the aqueous fraction could account for the antiHIV-RT activity. Work is in progress to identify the bioactive tannins present in it.

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