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Full Length Research Paper

In vitro activity of three selected South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase

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Crude extracts of three ethnobotanically selected medicinal plants were screened for activity against two functions of human immunodeficiency type 1 reverse transcriptase. Inhibition of the RNA-dependent DNA polymerase activity was evaluated by measuring the degree of incorporation of methyl-3H thymidine triphosphate using polyadenylic acid.oligodeoxythymidylic acid as a template primer. Ribonuclease H activity was evaluated by measuring the extent of degradation of a radiolabelled RNA in an RNA/DNA hybrid by reverse transcriptase in the presence of test substance. The methanol extract of the leaves of *Terminalia sericea* (Combretaceae) was found to strongly inhibit the polymerase (IC_{50} = 7.2 µg/ml) and the ribonuclease H (IC_{50} = 8.1 µg/ml) activities. Isolation and characterization of a possible active molecule is warranted.

Key words: HIV-1 reverse transcriptase; inhibition; crude extracts; medicinal plants; *Terminalia sericea*; South Africa.

INTRODUCTION

Reverse transcriptase, protease and fusion inhibitors currently used in the treatment of human immunodeficiency virus (HIV) infection are beneficial in improving the quality of life of HIV/AIDS patients. Nevertheless, the development of resistance, appreciable levels of toxicity, high cost, unavailability and above all the lack of a curative effect are their major shortcomings (Pomerantz and Horn, 2003). Consequently, the search for better anti-HIV therapeutic agents continues. A prominent approach to achieve more useful agents against HIV is the designing of novel antagonists to HIV enzymes and the development of inhibitors against other viral targets based on molecular modelling. However, another focus has been on natural sources, particularly plants, as a source of potent anti-HIV agents (De Clercq, 2000).

Several studies have described the inhibitory properties of medicinal plants on different targets in the life cycle of HIV (Vlietnick et al, 1998; Asres et al., 2001; Chang and Woo, 2003). In these studies, activity against the HIV enzymes reverse transcriptase (RT), protease, integrase, and anti-fusion properties of crude plant extracts and/or isolated principles were evaluated. Indeed, the search for compounds capable of halting HIV replication has led to the isolation of known and novel molecules, a few of which have entered clinical trials (De Clercq, 2000; Kong et al., 2003).

South Africa has a rich diversity of medicinal plants and the use of herbs is widespread. With about 5 million HIV infected people, a good proportion of patients for

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Table 1. Ethno-medical information of three South African medicinal plants used in the treatment of HIV/AIDS patients.

Plant identify (Voucher number)	Common names	Use in traditional medicine
Bridelia micrantha Hochst. Baill Euphorbiaceae (BP 03-1)	Munzere (t), Metzeeri (e)	Diarrhoea, stomach ache, sore eyes.
Combretum molle R. Br. Ex G. Don Combretaceae (BP07-1)	Mugwiti (t), Velvet bushwillow (e)	Fever, abdominal pains, convulsion, worm infections
Terminalia sericea Burch. Ex Dc (BP09)	Mususu (t) Silver cluster leaf (e)	Cough, skin infections, diarrhoea

e = Common name in English
t = Common name in Tshivenda

traditional and financial reasons, seek treatment from traditional healers who administer preparations from a variety of plants. Anecdotes of the therapeutic value of South African herbs in HIV/AIDS are not lacking (Morris, 2002; DOH, 2003). AIDS is a syndrome comprising a dysfunction of the immune system and exacerbated by opportunistic infections of bacterial, fungal, protozoan or viral etiology. The therapeutic benefit of any herbal preparation in the HIV/AIDS condition could be as a result of the inhibition of viral replication, invigorating the immune system, or having inhibitory properties against opportunistic infections. There is a dearth of experimental data on the effects of South African medicinal plants on HIV despite the administration of plant-based decoctions and concoction to HIV/AIDS patients in the country (Motsie et al., 2003).

Human immunodeficiency virus type 1 RT, an essential enzyme in viral replication performs three principal functions: Firstly, the polymerase domain transcribes viral genomic RNA to viral DNA, a process referred to as the RNA-dependent DNA polymerase (RDDP) activity. Secondly, in the course of reverse transcription an intermediary RNA/DNA hybrid is formed. RT through its ribonuclease H (RNase H) domain degrades the RNA component of the hybrid. Thirdly, RT carries out DNA-dependent DNA polymerization activities, producing complementary DNA strands. The completion of each of these processes is required for the formation of a competent viral DNA capable of integrating into the genome of the infected cell. In the present report, crude extracts of three ethnobotanically selected plants used in the Limpopo Province of South Africa by traditional healers in the treatment of AIDS were evaluated for biological activity against HIV-1 RT RDDP and RNase activities. The cytotoxicity of the extracts was also determined.

MATERIALS AND METHODS

Selection of medicinal plants

In selecting medicinals plants, four traditional healers were asked to describe plants they use in treating individuals presenting with two or more combinations of weight loss, intermettent fever, persistent cough, diarrhoea and skin rashes (CDC, 1993). Three of the healers believed they treat AIDS patients because this has been disclosed to them by the patients themselves following clinical evaluation in 'western' medicine. Based on this approach three plants commonly used by the healers were selected: the leaves of *Terminalia sericea*, and the roots and stem-bark of *Bridelia micrantha* and *Combretum molle*. The plants were identified by Mr Peter Tshisikawe of the Botany Unit, University of Venda. Thohoyandou, South Africa were voucher specimens have been deposited. For conservation purposes leaves and stem-bark were harvested for used in this study. Plant identification and ethnomedical information are presented in Table 1.

Preparation of crude plant extracts

Leaves or stem-barks were washed in distilled water, chopped into small pieces and allowed to dry at room temperature in the shade for at least two weeks. Dried material was ground to powder. Aqueous extracts were prepared in line with traditional medicine procedures. Approximately 200 g of ground material was infused in 1 l of hot distilled water and left overnight on a rotating platform. This was filtered through a cheese-cloth and then under pressure through a qualitative Whatman filters paper No.3 (W&R, England, UK). The filtrate was evaporated to dryness in a rotatory evaporator (Rotavapor R-114, Buchi, Switzerland) at 60°C. Methanol extracts were similarly prepared as the aqueous extracts. However, plant material was soaked in distilled methanol (Labchem, Johannesburg, South Africa), and the filtrate was evaporated to dryness at 40°C. The crude extracts were stored at 4°C in the dark until used.

HIV-1 Reverse transcriptase assays

Activity of plant extracts were investigated for their ability to inhibit the RNA-dependent-DNA polymerase (RDDP) and the ribonuclease H (RNase) activities of HIV-1 RT. Recombinant HIV-1 RT used in these experiments was obtained from Professor Simon Litvak (Laboratoire de Réplication des Génomes Eucaryotes et Rétroviraux, CNRS, Université de Bordeaux II, Bordeaux, France). The enzyme consists of the p66 and p51 subunits (Sallafranque-Andreola et al., 1989).

Evaluation of crude plants extracts against RDDP activity

The inhibition of RDDP activity was measured by evaluating the incorporation of methyl-3H thymidine triphosphate (Methyl [3H] TTP) by RT using polyadenylic acid-oligodeoxythymidilic acid (polyA-dT) as a template-primer in the presence and absence of plant extract (Sallafranque-Andreola et al., 1989**).**

Preparation of PolyA-dT template- primer PolyA-dT of final

optical density of 2.4 was constituted by mixing

8.3 µl of poly ribosomal adenylic acid (OD 120) (Sigma), 10 µl of oligodeoxythymidlic acid (Sigma) (OD 20), 5 µl of 1 M Tris pH 7.5 (Euromedex, France) and 476.7 µl of distilled water. This gives a final optical density of 2.0 and 0.4 for polyA and oligo dT respectively, and a final concentration of 10 mM for Tris pH 7.5.

RDDP inhibition assay

Activity of extracts was investigated in a 50 µl reaction mixture containing 50 mM Tris HCl (pH 7.9), 10 mM dithiothreitol, 5 mM MgOAc, 80 mM KCl, 2 µM dTTP, 0.5 uCi [methyl-3H] dTTP (70Ci/mmole), 20 µg polyA-dT (5:1), 0.02 µM of RT. Prior to use, the aqueous extracts were dissolved in distilled water, and methanol extracts were dissolved in dimethylsulphoxide (DMSO) (Merck). The final concentration of DMSO was 5%. Negative controls without extract, were set up in parallel. Reaction tubes were incubated at 37°C for 10 min and the reaction was stopped by adding 3 ml of a 0.1 M sodium pyrophosphate/10% trichloroacetic acid cold solution. Radioactive polymerized residue collected on cellulose nitrate transfer membranes (0.45 microns, Whatman) was dried and immersed in scintillating fluid (Ultima Gold, Packard Bioscience). Radioactivity was measured in a Wallac 1409 scintillating counter and was expressed as counts per minute (CPM). Percentage inhibition was calculated as 100 – [(CPM with extract/CPM without extract) x 100]. Reactions were carried out in duplicate for each of three independent determinations. Azidothymidine triphosphate (AZT-TP) was used as a positive control.

Synthesis of [3H] RNA/DNA hybrid

RNA/DNA hybrid, the substrate of RNase H, was synthesized as earlier described (Andreola et al., 1993). Essentially, RNA/DNA hybrid was prepared in a 50 µl reaction volume containing 0.01 mg/ml calf thymus single stranded DNA, (Sigma-Aldrich), 1 U *E. coli* RNA polymerase (Boehringer), 50 mM Tris HCl, 5 mM dithiothreitol, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM each of ATP, GTP, CTP (Roche) and [3H]UTP (20 µCi). The mixture was incubated at 37°C for 1 h, followed by the addition of 0.5 mM UTP for further transcription for 10 min at 37°C. The radiolabelled RNA/DNA hybrid was stored at -20°C until used.

RNase H inhibition assay

Plant extracts were tested in a 50 µl reaction volume containing 50 mM Tris HCl (pH 7.8), 60 mM KCl, 5 mM $MgCl_2$, 1 µl of [3H] RNA/DNA hybrid (20,000 CPM) and 0.02 µM of RT. Reaction was incubated for 15 min and then treated as for the RDDP assay. Controls comprised RNA/DNA hybrid devoid of extract and RT, and RNA/DNA hybrid with RT. Anti RNase H activity was evaluated by measuring the degree of degradation of the 3H-labelled RNA strand in a RNA/DNA hybrid by RT in the presence of the test substance. Percentage inhibition of RNase H activity was calculated as [1 - (CPM of RNA/DNA hybrid without extract – CPM of RNA/DNA hybrid with test substance) / (CPM of RNA/DNA hybrid without extract – CPM of RNA/DNA hybrid with RT)] x 100. Reactions were carried out in duplicate for each of three independent determinations. A DNA aptamer (ODN 93) was used as a positive control (Andreola et al., 2001a).

Cytotoxicity assay

Cytotoxicity of crude extracts was determined in a human epitheloid cervical carcinoma cells line transfected with the CD4 molecule (HeLaP4) as earlier reported by Andreola et al. (2001a), with some modification. Briefly, a 96 flat-bottom well microtitre plate was seeded with 12,000 cells in a total volume of 200 µl Dulbecco's minimum essential medium containing 10% fetal calf serum and gentamicin (45 µg/ml). This was incubated for 24 h at 37°C in 5% CO_2 humidified atmosphere. Culture medium was discarded and a two-fold serial dilution of test substance was done in a total volume of 200 µl for a concentration range of 600 – 0.59 µg/ml. The plate was incubated for 48 h. Prior to testing, aqueous extracts were dissolved in distilled water, while methanol extracts were initially dissolved in 50% DMSO. The final DMSO concentration tested was below 1.6%. Controls consisting of triple wells containing cells and growth medium without extract, growth medium alone, and cells in growth medium with 2% DMSO were set up in parallel. Extract concentrations were evaluated in duplicate. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. This is a colorimetric method in which viable cells reduce a tetrazolium compound to a soluble formazan product. Cells and reagent were incubated for 3 h. Absorbance values were measured at 490 nm. The assay was performed twice. Fifty percent cytotoxic concentration (CC_{50}) was computed as the concentration of extract that reduced cell viability by 50% when compared to controls.

RESULTS AND DISCUSSION

The methanol extract of the leaves of *T. sericea* was observed to strongly inhibit the RDDP and RNase H functions of HIV-1 RT in a dose-dependent manner with IC50 values of 7.2 µg/ml and 8.1 µg/ml respectively. All the other extracts showed weak anti-RT properties with IC50 values > 18.0 µg/ml). In general, the methanol extracts were more inhibitory than the aqueous extracts. It was also observed that the anti-RDDP and anti- RNase H activities of the extracts were fairly comparable (Table 2). All the plant extracts were non-toxic to HeLaP4 cell line at a concentration of 600 µg/ml (data not shown).

In order to combat HIV, the causative agent of AIDS, enormous amount of human and material resources have been dedicated to research on compounds which can be developed as therapeutic agents. As part of our on-going programme investigating the antimicrobial properties of medicinal plants from the Venda Region of South Africa, a region where traditional medicine is striving (Arnold and Gulumian, 1984), the aqueous and methanol extracts of three selected plants used in the treatment of AIDS-related pathologies by traditional healers in the Venda Region were screened against HIV-1 RT, an enzyme essential for viral replication.

We observed that the methanol extract of the leaves of *T. sericea* showed the strongest inhibition against HIV-1 RT RDDP and RNase functions. Although the molecules that could be responsible for this activity were not investigated, tannins and triterpenes have been isolated from the leaves of this plant (Aganga and Adogla-Bessa, 2000; Khanal et al., 2001; Rode et al., 2003). Elsewhere, both condensed and hydrolysable tannins, and triterpenes have been shown to be strong inhibitors of

Table 2. Inhibition of HIV-1 RT RDDP and RNase H activities by three South African medicinal plants.

Plant	Plant part investigated	Extract type	% yield (w/w)	RDDP function		RNase H function	
				% inhibition[a]	IC50(µg/ml)[b]	% inhibition[a]	IC50 (µg/ml)[b]
B. micrantha	Leaves	Aqueous	1.2	68.2 ± 3.2	34.6	71.3 ± 1.6	27.9
		Methanol	3.6	77.1 ± 2.7	23.5	67.1 ± 1.0	18.9
C. molle	Stem-bark	Aqueous	1.3	58 ± 1.1	81.3	64.6 ± 1.3	79.1
		Methanol	6.8	85.3 + 2.1	20.3	79.1 ± 2.7	21.6
T. sericea	Leaves	Aqueous	2.2	74.2 ± 3.1	24.1	87.3 ± 2.2	18.5
		Methanol	7.2	98 ± 0.8	7.2	99.3 ± 2.5	8.1

[a]Percentage inhibition is given as the mean inhibition standard deviation of three independent determinations with an extract concentration of 100 µg/ml.
[b]IC50 is the concentration of extract required to reduce the activity of HIV-1 RT by 50%. The value was derived by extrapolation from concentration-activity regression curves.

HIV-1 RT *in vitro* (Tan et al., 1991; Notka et al., 2003), with potential specificity of action (Zhu et al., 1997).

It was also observed that the methanol extract of *T. sericea* inhibited the RDDP and RNase H activities by fairly comparable degrees. The reason for this is not clear. However, the RDDP activity is mediated by the polymerase domain located on the N-terminal of the HIV-1 RT molecule, while the RNase H activity is mediated by the p15 component located on the C-terminal. In the course of enzyme activity these two domains of the molecule interact (Andreola et al., 2001b). This interaction may explain the double antagonistic effect of the extract on HIV-1 RT.

In addition, although many molecules including RT inhibitors in current clinical use, capable of inhibiting the polymerase activity of HIV-1 RT have been identified, not many antagonists of the RNase H activity have been described. The RNase H domain should be an attractive target to arrest viral proliferation, since point mutations in the RNase H domain of RT induce significant decrease in viral replication (Tarrago-Litvak et al., 2002). Whether the component responsible for the anti-HIV-1 RT activity observed in this investigation for *T. sericea* is broad spectrum or non-specific could only be determined by its isolation and evaluation of its mode of action.

In previously reported studies, extracts obtained from *T. sericea* have been shown to have strong antimicrobial effects against *Stapylococcus aureus* and *Candida albicans* (Fyhrquist et al., 2002; Nakamura et al., 2004). *S. aureus* is implicated in skin infections, while infection with *C. albicans* manifests as oral thrush and vulvovaginitis. The prevalence of *S. aureus* and *C. albicans* in HIV/AIDS as opportunistic infections is well documented (Miller et al., 2003; Bertagnolio et al., 2004; Lattif et al., 2004). It is also possible that the beneficial effects of decoctions made from *T. sericea* on HIV/AIDS patients may be linked to its inhibition of common opportunistic infections of bacterial or fungal etiology. In another vein, decades of use of a particular plant may point to its non-toxicity. However, there have been reports of human poisoning due to the ingestion of decoctions made from commonly used medicinal herbs (Hamouda et al., 2000; Onen et al., 2002). Consequently, it is important to screen commonly used herbal medicine for potential toxicity. Herein, the aqueous and methanol extracts of the leaves of *B. micrantha*, *T. sericea* and the stem-bark of *C. molle* were found to be non-toxic to a HeLaP4 cell line at 600 µg/ml. In conclusion, due to the observed activity of the methanol extract of *T. sericea* against HIV-1 RT, we are employing a bioassay-guided fractionation protocol to isolate and chemically characterize the molecule responsible for its activity.

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