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In vitro anti-HIV-1 properties of ethnobotanically selected South African plants used in the treatment of sexually transmitted diseases

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ABSTRACT

Ethopharmacological relevance: The plants selected in this study are used traditionally in the treatment of sexually transmitted diseases and traditional healers interviewed claimed these plants can also help AIDS patients.
Aim: To evaluating the *in vitro* anti-HIV properties of selected plants in various bioassays.
Materials and Methods: The extracts were evaluated for their inhibition against α-glycohydrolase, reverse transcriptase and viral proteins (NF-κB and Tat) which play a significant role in the HIV life cycle.
Results: Terminalia sericea extract (IC_{50} = 92 mg/ml) exhibited a considerable α-glucosidase inhibitory activity which was better than acarbose (IC_{50} = 131 mg/ml) under our assay conditions. In the reverse transcriptase assay, *T. sericea* also showed good inhibitory activity (IC_{50} = 43 mg/ml), which was higher than that of the reference drug, Adriamycin (IC_{50} = 100 mg/ml). The ethyl acetate extract of *Elaeodendron transvaalense* exhibited the most potent inhibitory activity in both the NF-κB and Tat assays with inhibitory activity of 76% and 75% respectively at a concentration of 15 mg/ml. The acetone and chloroform extracts of *E. transvaalense* and *Zanthoxylum davyi* also showed good activity in the NF-κB and Tat assays.
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1. Introduction

Plant products have attracted attention as possible anti-HIV (human immunodeficiency virus) drugs targeted on the different steps of the viral life cycle, such as viral attachment and entry and on essential enzymes that play a role during viral genome transcription (Matsuse et al., 1999). The HI virus infects CD4+ T lymphocytes and macrophages and its genetic material is integrated into the infected cell genome. Upon integration the virus remain transcriptionally silent and this allows the infected cells to escape currently used antiretroviral drugs. In latently infected cells, viral transcription can be reactivated by various stimuli, including, phorbol esters and cytokines (Marcello et al., 2004). When cells are activated, transition from latency to HIV expression occurs and requires the converted action of cellular transcription factors and regulatory HIV proteins (Bedoya et al., 2005). Among these proteins are the cellular transcription factor, NF-κB and HIV Tat which are required for efficient HIV replication. These proteins regulate the post-integration phase of the viral cycle, which preferentially occurs in activated cells on the long terminal repeat promoter (LTR). The viral regulatory proteins and cellular factors represent potential targets that should be considered in the search of anti-HIV agents, because they determine the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (Sancho et al., 2004).

Because of a persistent and urgent need for an anti-HIV drugs, interest in the anti-HIV activity of traditional medicinal plants has now gained momentum. Because of their relatively low cost, plants have been increasingly explored for production of biomedicine and vaccines (Karasev et al., 2005). Numerous plant-derived substances including phenylcoumarins and plant proteins have shown good anti-HIV activity that can be related to inhibition of NF-κB and Tat proteins (Akesson et al., 2003; Reddy et al., 2004; Marquez et al., 2005).

This study was aimed at evaluating the *in vitro* anti-HIV properties of ten ethnobotanically selected South African plants by various bioassays.

2. Materials and methods

2.1. Plant material

All the plant materials (roots and stem bark) collected were selected based on their traditional uses against sexually transmitted diseases (syphilis, gonorrhea, herpes as well as HIV). The plant

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Table 1
Medicinal plants investigated for anti-HIV activity

Species	Family	Part used for STD's	Voucher no.	Other ethnobotanical information
Anredera cordifolia (Ten.) Steenis	Basellaceae	Stem tubers	Smit 085981	Pain, inflammation (Tornos et al., 1999)
Clerodendrum glabrum E. Mey var. glabrum	Lamiaceae	Roots	Van Wyk. 51839	Malaria (Clarkson et al., 2004)
Elaeodendron transvaalense (Burtt Davy) R.H. Archer	Celastraceae	Stem bark	Tshikalange 092524	Stomach ache, fevers, diarrhea (Van Wyk et al., 1997)
Polianthes tuberose L.	Agavaceae	Roots	E.T 29	Ornamental (Huang et al., 2001)
Rauvolfia caffra Sond.	Apocynaceae	Stem bark	Hemm 39291	Diarhoea, abdominal complaints (Palgrave, 1977)
Rotheca myricoides (Hochst.) Vatke	Lamiaceae	Roots	Van Wyk. 45727	Malaria (Muregi et al., 2007)
Senna occidentalis (L.)	Fabaceae	Roots	Lubbe 075884	Malaria (Tona et al., 2004)
Senna petersiana (Bolle) Lock	Fabaceae	Roots	Van Wyk. 070978	Fevers, skin infections (Coetzee et al., 2000)
Terminalia sericea Burch. ex DC.	Combretaceace	Roots	Van Rensburg 38564	Diabetes, diarrhea (Moshi and Mbwambo, 2005)
Zanthoxylum davyi (I.Verd.) P.G. Waterman	Rutaceae	Roots	Lubbe 078130	Chest pains, wounds, toothache, coughs (Tarus et al., 2006)

material of all studied plants are traditionally dried, pounded and drunk as infusion except for *Elaeodendron transvaalense* and *Zanthoxylum davyi* which can be taken either as infusion or decoction. Information on the use of these medicinal plants was gathered through interviews with traditional healers and literature review. The plants investigated (Table 1) were collected from Venda in the Limpopo Province (South Africa). Voucher specimens were prepared and identified at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria. Colleted plant material were air-dried in the shade at room temperature and then ground with a grinder into a fine powder which were stored in airtight containers at room temperature.

2.2. Extract preparation

Dried powdered plant materials were extracted with several solvents (chloroform, ethyl acetate, water and 70% acetone). Thirty gram of powdered material were extracted twice for 2 h with 300 ml of solvent and filtered. The extracts were then concentrated to dryness under reduced pressure and the residues freshly dissolved in an appropriate solvent on the day that the bioassay was done.

2.3. Bioassays

2.3.1. Glycohydrolase enzymes

The inhibition of the glycohydrolase enzymes, α-glucosidase and β-glucuronidase were determined in the presence of their substrates, ρ-nitrophenyl-α-D-glucopyranose and ρ-nitrophenyl-β-D-glucuronide respectively in a 96-well microtitre plate in a colorimetric enzyme based assay (Collins et al., 1997). The substrates and enzymes were dissolved in 50 mM morpholinol Thanesulfic acids–NaOH, pH 6.5. The assay was calibrated relative to the enzyme concentration of 0.25 μg enzyme used per assay. Acarbose, an anti-diabetic drug used to treat type 2 diabetes mellitus and also an inhibitor of alpha-glucosidase was used as a positive control (Andrade-Cetto et al., 2008). To test enzyme inhibition, assays were performed as describe by Harnett et al. (2005). The extracts were tested at 200 μg/ml and the experiment was carried out in triplicate.

2.3.2. HIV reverse transcriptase (RT) assay

The effect of plant extracts on RT activity was evaluated with a non-radioactive HIV–RT colorimetric ELISA kit (Roche, Germany). The concentration of the extracts tested was 200 μg/ml. Adriamycin, an anticancer drug and also an inhibitor of viral reverse transcriptases was used as a positive control (Goud et al., 2003). The assay was carried out in triplicate.

2.3.3. Cell lines

MT2 cells were cultured in RPMI 1640 medium (Gibco BRL), containing 10% fetal bovine serum, 2 mM glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml). MT-2 cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere and splinted twice a week. The 5.1 cell line (obtained from Dr. N. Israel, Institut Pasteur, Paris, France) was maintained as MT2 cell line but the medium was supplemented with 100 μg/ml G418 (Gibco BRL). Both Hela-Tat-Luc and HeLa-Tet-ON cell lines were maintained in DMEM (Gibco BRL) in the presence of 100 μg/ml of hygromycin (Invitrogen) and 100 μg/ml of G418 (Gibco BRL). These cell lines were maintained at 37 °C in a 5% CO_2 humidified atmosphere and splinted when confluent.

2.3.4. 5.1 Cell line assay

To determine the anti-NF-κB activity of the selected extracts a NF-κB-dependent luciferase assay was used. The 5.1 cell line was a Jurkat-derived clone stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter. This promoter is highly dependent on NF-κB activation induced by TNFα. Therefore high expression of luciferase activity reflects NF-κB activation through the canonical pathway (Sancho et al., 2004).

The assay was performed as described by Marquez et al. (2005). Briefly, 5.1 cells were pre-incubated with increasing concentrations of the extracts for 30 min and then stimulated with TNFα (2 ng/ml) for 6 h. The cells were lysed in 25 mM Tris phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 953 following the instructions of the luciferase assay kit (Promega) and protein concentration was measured by the Bradford method (Marquez et al., 2005). The background obtained with the lysis buffer is subtracted in each experimental value and the specific transactivation is calculated as RLU/μg protein (relative light units) and the results were expressed as the percent of inhibition with 100% activity assigned to transcriptional activity induced by TNFα alone (Campagnuolo et al., 2005). The extracts were tested at 50 μg/ml and the active extracts were further tested at 25, 15, 5 and 1 μg/ml concentrations. Mesuol was used as a reference inhibitor of NF-κB activities and the experiment was repeated four times.

2.3.5. Hela-Tat-Luc assay

To identify potential anti-Tat extracts, another luciferase-based cell system (HeLa-Tat-Luc cells) was used. The Hela-Tat-Luc cells are stably transfected with the plasmid $pcDNA_3$-TAT together with a reporter plasmid LTR-Luc. Therefore the HIV-1 LTR is highly activated in this cell line as a consequence of high levels of intracellular Tat protein. Cells (10^5 cells/ml), seeded the day before the assay, were treated either with the CDK9 inhibitor DRB, as a positive control, or with the plant extracts. After 12 h, the cells were washed twice with PBS and the luciferase activity measured as indicated previously for 5.1 cells. The extracts were tested at 50 μg/ml and the active extracts were further tested at 15, 5 and 1 μg/ml concentrations. The experiment was repeated four times.

2.3.6. Hela-Tet-ON-Luc assay

Extracts considered to be active in both NF-κB (>50% inhibition) and Tat (>30% inhibition) assays, were subsequently evaluated by Hela-Tet-ON assay to discard nonspecific luciferase inhibitory activity (Sancho et al., 2004).

The cells (10^5 cells/ml) were seeded the day before the assay, and stimulated with doxycycline (1 μg/ml) in the presence or absence of the extracts for 6 h. The cells were washed twice in PBS, lysed and the luciferase activity measured as described (Sancho et al., 2004). Mesuol was used as a reference for specific mode of action. The extracts were tested at 50 μg/ml and the experiment was repeated four times.

2.4. Cytotoxicity assay

MT2 cells (10^5/ml) were seeded in 96-well plates in complete medium and treated with increasing doses of the extracts for 36 h. Samples were then diluted with 300 μl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 μg/ml). After incubation, cells were immediately analyzed by flow cytometry (Marquez et al., 2005). All the results were calculated as percentage of cell death by GraphPad software.

3. Results and discussion

In the *in vitro* assay of α-glucosidase, *Terminalia sericea* extract exerted the highest inhibitory activity (IC_{50} = 92 μg/ml), followed by *Senna petersiana* with IC_{50} value of 135 μg/ml. All the other extracts tested (*Anredera cordifolia*, *Clerodendrum glabrum*, *Elaeodendron transvaalense*, *Poliathes tuberose*, *Rauvolfia caffra*, *Rotheca myricoides*, *Senna occidentalis* and *Zanthoxylum davyi*) showed weaker (not significant) or no inhibition against α-glucosidase. *Terminalia sericea* exhibited a higher inhibitory activity than acarbose (IC_{50} = 131 μg/ml) under our assay conditions. In the studies done by Wansi et al. (2007), *Terminalia superba* stem bark extract exhibited a considerable α-glucosidase inhibitory activity and the active compounds were gallic acid and methyl gallate. It is also possible that the activity of *Terminalia sericea* found in this study might be attributed to similar or related compounds in *Terminalia superba*. In the β-glucuronidase assay, the most active extracts were *Senna petersiana* and *Terminalia sericea* which exhibited IC_{50} values of 87 and 92 μg/ml, respectively. There are published reports on other biological activities such as antimicrobial activities, but this is the first report on the inhibition of the β-glucuronidase enzyme by the plants selected in this study (Steenkamp et al., 2007; Tshikalange et al., 2005).

The crude extract of *Terminalia sericea* exhibited strong HIV-1 RT inhibitory activity with the IC_{50} value of 43 μg/ml while the other plant extracts did not inhibit the RT enzyme. Adriamycin exhibited lower inhibitory activity (IC_{50} = 100 μg/ml) than *Terminalia sericea*. Further studies need to be conducted in order to identify the active compound that might be responsible for inhibiting RT. In previous reported studies, extracts of *Elaeodendron transvaalense* roots and *Terminalia sericea* leaves have been shown to have some activity in HIV-1 RT RDDP and HIV-1 R RNase H assays (Bessong et al., 2005; Bessong et al., 2004). However in this studies stem bark of *Elaeodendron transvaalense* roots and *Terminalia sericea* roots were used. Eldeen et al. (2006) reported the isolation of the (terpenoid) anolignan B from *Terminalia sericea* root extract. This compound was first isolated from *Anogeissus acuminate* and was reported as a constituent acting with anolignan A to inhibit the HIV-RT enzyme (Eldeen et al., 2006). In other types of bioassays, biological activities of *Terminalia sericea* in other types of bioassays were mainly attributed to triterpenoids, saponins and tannins (Steenkamp et al., 2004; Bombardelli et al., 1974; Fyhrquist et al., 2006).

Both chloroform and ethyl acetate extracts of *Elaeodendron transvaalense* showed good inhibitory activity of 64% and 76% respectively at the lowest concentration tested (1 μg/ml) in the NF-κB assay (Table 2), where as acetone extracts of *Elaeodendron transvaalense* and *Zanthoxylum davyi* exhibited little or no activity. At the highest concentration (15 μg/m) acetone (54%), chloroform (73%) and ethyl acetate (75%) extracts of *Elaeodendron transvaalense* together with the acetone extract (54%) of *Zanthoxylum davyi* showed good inhibitory activity. Mesuol (positive control) exhibited a higher inhibitory activity (84.01%) than the tested plant extracts. All the plant extracts were also analysed for their anti-Tat activity in the HeLa-Tat-Luc assay. Chloroform and ethyl acetate extracts of *Elaeodendron transvaalense* showed a high Tat inhibitory activity of greater than 70% at 15 μg/ml (Table 2). Acetone extract of *Elaeodendron transvaalense* demonstrated lower activity (>50%), while *Zanthoxylum davyi* exhibited a moderate activity (50%) at 50 μg/ml. Those extracts showing anti-NF-κB and anti-Tat activity were also found to be specific in the HeLa-Tet-On assay and did not cause a significant cytotoxicity in the MT2 cell line. Further studies including isolation of active compounds and a deeper insight to determine the mechanism of action is required for assessing potential anti-HIV lead.

The active extracts were also analysed for cytotoxicity (Table 2) to determine whether the activity was due to toxicity. The results showed that, these extracts did not cause significant cell death in the MT2 cell line. The acetone, ethyl acetate and chloroform extracts of *Elaeodendron transvaalense* showed lower cell death percentages after 36 h at the highest concentration tested (15 μg/ml). The acetone extract of *Zanthoxylum davyi* showed little toxicity of 2.4% cell death. These results indicate that, at the concentrations tested, anti-NF-κB and anti-Tat activity was not due to cellular toxicity.

Many attempts at screening traditional medicine have been made in search for anti-HIV active agents from natural (Hussein et al., 1999). However the South African medicinal plants studied in this report have not been investigated for their antiviral activity through the inhibition of both NF-κB and Tat proteins. In this first report extracts of *Elaeodendron transvaalense* and *Zanthoxylum davyi* showed *in vitro* anti-HIV properties through the

Table 2
Results of anti-HIV evaluations for plant extracts that showed activity in the 5.1 NF-κB, HeLa-Tat-Luc and HeLa-Tet-ON assays

Plant	Extracts	Yields (%, w/w)	5.1 NF-κB (inhibition %)			HeLa-Tat-Luc (inhibition %), concentration (μg/ml)			HeLa-Tet-ON	Toxicity (cell death %)
			1	5	15	1	5	15	50	15
Elaeodendron transvaalense	70% Acetone	12	0.0	45.0	54.0	0.0	22.0	43.0	S[a]	22.7
Elaeodendron transvaalense	Chloroform	0.9	57.0	64.0	73.0	28.0	66.0	76.0	S	27.6
Elaeodendron transvaalense	Ethyl acetate	8	76.0	72.0	75.0	63.0	66.0	75.0	S	17.1
Zanthoxylum davyi	70% Acetone	14	34.0	48.0	54.0	1.4	25.0	50.0	S	2.4
Mesuol			NT	NT	84.0	NT	NT	72.3	S	24.3

Rest of the values are percentages of inhibition. NT: not tested.
[a] S: specific (inhibition <15%).

inhibition of both NF-κB and Tat proteins. The mode of action was specific and the active extracts being less toxic. The use of plant extracts or plant derived synthetic compounds targeting cellular proteins required for efficient HIV-1 replication and transcription has opened new avenues for scientific research in the management of AIDS. According to Marquez et al. (2005), plant-derived antiviral compounds interfering with HIV-1 LTR promoter regulatory proteins are unlikely to generate drug-resistant HIV strains if proven useful for patients. Further studies to determine the chemical identification of the active constituents in *transvaalense*, *Terminalia sericea* and *Zanthoxylum davyi* are in progress.

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