Evaluation of the HIV-1 reverse transcriptase inhibitory properties of extracts from some medicinal plants in Kenya.

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SUMMARY

Extracts from twenty two medicinal plants popularly used in preparing traditional remedies in Kenya were screened for activity against the HIV-1 reverse transcriptase. The screening procedure involved the use of tritium labeled thymidine triphosphate as the enzyme substrate and polyadenylic acid.oligodeoxythymidylic acid [poly(rA).p(dT)$_{12-18}$] as the template primer dimer. Foscarnet was used as a positive control in these experiments. At a concentration of 100µg/ml, extracts from eight of these plants showed at least 50 per cent reverse transcriptase inhibition. This activity was abitrarily considered as significant. This indicates that there is the probability that some antiretroviral compounds could be identified and isolated from materials from these plants.

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Introduction

The current therapy for the disease human immunodeficiency virus/AIDS (HIV/AIDS) has been focused on the use of antiretroviral drugs. However, the available antiretroviral drugs at present have fallen short of expectation in many ways. There is the problem of rapid development of resistance of the HIV virus to the drugs and the high cost of these drugs thereby making it inaccessible to those who badly need them in the developing countries. Therefore, there is still an urgent need to search for new agents and novel compounds that can overcome the shortcomings of these drugs.

There have been frequent reviews on the anti-retroviral agents in the clinic and in development. In one of these reviews [1] a concise account was presented on the usefulness of the non-nucleoside reverse transcriptase inhibitors. It would be logical to assume that the compounds of plant origin that would have anti-retroviral properties would most likely be non-nucleosides [2]. Indeed, the plant-derived compounds reported in the literature [3-8] so far with inhibitory properties on reverse transcriptase are non-nucleosides. However, the possibility of finding nucleoside HIV reverse transcriptase inhibitors of plant origin should not be ruled out. This paper describes the results of the screening of some extracts from medicinal plants that are frequently used in the preparation of traditional remedies for other diseases conditions. Although there is no reported evidence that these plant materials have been used, even traditionally, in the treatment of HIV/AIDS, it was felt that

the high frequency of use of these plants in other traditional remedies puts them on the priority list for investigation as potential sources of antiretroviral agents.

Materials and Methods

1. Preparation of plant extracts

The medicinal plants were collected from their natural habitats in the different geographical zones of Kenya. The plants were botanically identified by a specialist botanist who was part of the collection team. Voucher specimens are on deposit at the East African Herbarium. The stem and root barks were removed from the wood and cut into small pieces while the leaves were separated from the small branches. The materials were then spread on mats in the laboratory and allowed to dry for about two weeks at room temperature (~ 25^0 C.) and then powdered in a laboratory grinder.

The plant extracts were prepared as hot water extracts (unless otherwise stated) and then freeze dried. The preparation of the hot water extract involved boiling an aliquot of the dried powdered plant material with water for one hour. The hot extract was then filtered through cotton gauze and allowed to cool. The liquid extract was divided into 200 ml aliquots and placed in 500 ml round bottomed flasks. The aqueous extract was then frozen on the inside wall of each flask by rotating the flask in a solid carbon dioxide/acetone mixture. The flask containing the frozen extract was then attached to the manifold of an Edwards Modulyo Freeze dryer. A total of 1.2 litres distributed in six 500 ml flasks could be freeze dried at any one time and would take 48 to 72 hours to obtain completely moisture free material. This material was then stored in tightly screw capped bottles, and could be preserved for a considerable length of time.

Fractionation of some extracts

In a typical example the powdered stem bark of *P. africana* (KM 56) was extracted into methanol and dried under rotary evaporator. The dried extract (3.0 g) was loaded on to a column packed with Sephadex LH-20. Elution was carried out isocratically using methanol as the eluent at a rate of 0.3 ml / minute. Fractions of 20 ml were collected and purity monitored on TLC (Solvent; MeOH/$CHCl_3$/ H_20/AcOH, (50:34:6:1) with Vanillin-H_2SO_4 and Ferric ferricyanide as spray reagents. Similar fractions were pooled together yielding a total of 7 fractions. These fractions were dried and coded as KM 61, KM 62, KM 63, KM 64, KM 65, KM 66 and KM 67.

Typically the alkaloidal fractions were obtained as follows: The plant part was extracted into methanol and the extract dried through a rotary evaporator. The dried extract (25 g) was dissolved with 2% acetic acid (200 ml) and partitioned with chloroform (200 ml). The aqueous portion was adjusted to pH 9 with 30 % Ammonium Hydroxide, and then extracted with chloroform (500 ml). The chloroform fraction was dried yielding the alkaloidal fraction. Alkaloids were detected on TLC using Dragendorrff's spray reagent.

The preparation of a test solution involved weighing 10 mg of the dried material into a 10 ml volumetric flask and dissolving it in double distilled water and making it up to volume. The solution (1 mg/ml) was then centrifuged for 5 min to remove particulate matter.

2. Foscarnet solution.
   Foscarnet (Phosphonoformic acid sodium salt, Sigma) was used as a positive control. A stock solution containing 2 mg/ml was prepared in double distilled water. The stock solution was diluted with double distilled water to give a working solution of 2 µg/ml concentration and 5 µl was taken for the reaction in each experiment.

3. [^{3}H Methyl]thymidine 5'-triphosphate (Specific activity 30.0 Ci/mMol, Amersham International, plc). The stock solution of the radiolabeled material with concentration of 1 mCi/ml was diluted with double distilled water to give a working solution containing 0.1 µCi/µl.

4. Reverse transcriptase enzyme. The HIV-1 reverse transcriptase enzyme was obtained from Boehringer Mannheim Biochemica, Germany.

5. Poly (rA).p(dT)$_{12-18}$ sodium salt was obtained from Pharmacia Biotec.

6. Analytical grade reagents: 1M tris buffer pH 8.0; 2 M KCl; 200 mM magnesium chloride; Dithiothreitol.

7. Assay for reverse transcriptase activity.

Assay procedure.
A 50 µl reaction cocktail buffer was prepared as follows: 1 M Tris-HCl (pH 8.) 2.0 µl* (40 mM); 2 M KCl, 1.0 µl* (40 mM); 200 mM $MgCl_2$, 2.5 µl* (10 mM); 200 mM Dithiothreitol, 2.0 µl* (8 mM); 200 µg/ ml Poly(rA).p(dT)$_{12-18}$ sodium salt, 12.5 µl* (50 µg/ml); 0.1 µCi/µl [^{3}H]dTTP, 5.0 µl* (10 µCi/ml); 50-100 µg/ml test or ref. Sample, 5.0 µl*; H_2O,15.0 µl* . The volumes indicated with asterick * should give a total volume of 45 µl. This reaction mixture was kept on ice and then followed by adding the reverse transcriptase enzyme (5 µl) to initiate the reaction and thus bringing the reaction mixture to 50 µl. The reaction mixture was then incubated at 37^0 C. for 10 minutes. The reaction was terminated by taking a 15 µl aliquot and spotting it onto a 10 mm diameter paper disc and immediately immersing it in 50 ml of ice-cold solution of 5% trichloroacetic acid. The paper disc was washed twice in 50 ml ice-cold 5% trichloro acetic acid solution during 15 minutes on a rotatory shaker and changing the acid solution after each wash. The paper disc was then washed twice with 50 ml absolute ethanol on a rotary shaker for 5 minutes each time. The filter paper disc was then removed from the ethanol and air dried at 25^0 C. for 30 minutes. The filter paper disc was then transferred to a 5 ml vial containing 2 ml scintillation cocktail (NBCS 104, Amersham International, plc). The activity on the filter paper disc was then counted using a liquid scintillation counter (Beckman).
The counts per minute were then converted into per cent inhibition as follows:

$$\% \text{ Inhibition} = \frac{(\text{RT-Control CPM} - \text{Blank CPM}) - (\text{Sample CPM} - \text{Blank CPM})}{(\text{RT-Control CPM} - \text{Blank CPM})} \times 100$$

A preliminary reverse transcriptase enzyme concentration was determined by carrying out the assay on a fixed concentration of an inhibitor with varying concentrations of the enzyme. A plot of the enzyme concentration versus per cent inhibition gave a sigmoid curve of which a point on the straight line portion corresponded to the optimal enzyme concentration. This enzyme concentration was used for the particular batch of enzyme for the screening of the plant extracts.

Results

Table 1 presents a total summary of the enzyme inhibitory activities of the samples investigated for their action against HIV-1 reverse transcriptase. The values given are representative of each sample, giving an average of several experiments. It is considered to present the results simply without statistical presentations because the number of experiments carried out on the samples varied and the main objective at this level was to identify the most promising plant samples. At this stage therefore, parametric statistics was considered unnecessary since these samples were materials of undetermined composition. Arbitrary concentrations of 100 µg/ml and 50 µg/ml were used to determine the level of activity and those samples with high HIV-1 inhibitory activity were considered the most promising.

Table 2 presents the most promising results extracted from Table I and gives information on the botanical families of the plants. A high degree of selectivity in anti-HIV-1 activity was observed among the extracts. This is significant in view of the fact that these samples were total plant extracts that were being examined for enzyme inhibitory activity. This selectivity in activity was also shown in the plant botanical families that had plants yielding extracts with significant activity. In Table II 10 medicinal plants in six plant families with such a profile are given:

1. *Maytenus buchananii* (Celastraceae)
2. *Maytenus senegalensis* (Celastraceae)
3. *Acacia mellifera* (Leguminosae)
4. *Erythrina abyssinica* (Leguminosae)
5. *Azadirachta indica* (Meliaceae)
6. *Leptotrichilia* sp. (Meliaceae)
7. *Melia azedarach* (Meliaceae)
8. *Myrica salicifolia* (Myricaceae)
9. *Prunus africana* (Rosaceae)
10. *Grewia mollis* (Tiliaceae)

The results indicate that these extracts can be phytochemically examined.

Table 3 gives results of preliminary phytochemical screening and chromatographic fractionation of the extracts from one of the promising plants, Prunus africana (Rosaceae). This result indicates that the extract had a high content of polyphenolic compounds, some terpenoids and no alkaloidal substances. It would be tempting to suggest that the anti-HIV reverse transcriptase activity could be ascribed to the polyphenolic content of the extract. An example of this is presented in Table 2 where the results of chromatographic fractionation is presented on the extracts from *Prunus africana* stem bark. Item Nos. 15 through 26 in Table II illustrates the effects of chromatographic fractionation and the use of the in vitro reverse transcriptase inhibitory assay model to monitor such a fractionation.

Table 1: Inhibition of HIV-1 Reverse Transcriptase by medicinal plant extracts.

Serial. No.	Sample Code		% Inhibition	% Inhibition
		Plant name & part used	100 µg/ml	50 µg/ml
1	KM 01	*Toddalia asiatica* (L.) Lam.: root	-	25.43
2	KM 02	*Azadiracta indica* A.Juss.: leaves	70.93	51.79
3	KM 03	*Toddalia asiatica* (L.) Lam: root	39.48	23.41
4	KM 04	*Albizia gummifera* (J F Gmel.) C A Sm: stem bark, alkaloid fraction	42.97	32.78
5	KM 05	*Albizia gummifera*(J F Gmel.) C A Sm: stem bark MeOH extract	39.65	1.77
6	KM 06	*Albizia gummifera*(J F Gmel.) C A Sm: stem bark, water extract	20.82	4.43
7	KM 07	*Maytenus buchananii* (Loes.) Wilczek.: roots	89.65	68.76
8	KM 08	*Maytenus buchananii* (Loes.) Wilczek.: stem bark	99.16	95.22
9	KM 09	*Maytenus senegalensis* (Lam.) Exell, roots	99.83	94.09
10	KM 10	*Tabernaemontana stapfiana* Brizten. roots	0.0	0.0
11	KM 11	*Azadirachta indica* A.Juss.., stem bark	96.95	89.01
12	KM 12	*Azadirachta indica* A.Juss.., stem wood	96.20	81.20
13	KM 14	*Typha domingensis* Pers.ash extr.	9.96	7.18
14	KM 15	*Melia azedarach* L. leaves	99.89	45.65
15	KM 16	*Warburgia ugandensis* Sprague stem bark	92.13	60.86
16	KM 19	*Erythrina abyssinica* D C stem bark	100.17	15.89
17	KM 21	*Prunus africana* (Hook f.) Kalkm stem bark	99.29	71.23
18	KM 24	*Zanthoxylum chalybeum* Engl. root bark	34.77	26.26
19	KM 25	Alkaloidal fraction from KM 24	44.66	26.17
20	KM 26	*Kigelia africana.* (Lam.) Benth. fruit	6.93	5.21
21	KM 27	*Rhamnus staddo* A.Rich roots	38.06	34.49
22	KM 29	*Myrica salicifolia* A.Rich. root bark	90.23	68.79
23	KM 30	Onion water extract	2.50	-
24	KM 31	*Acacia mellifera* (Vahl) Benth. stem bark	99.25	84.81
25	KM 32	*Azadirachta indica* A.Juss. root bark	59.64	35.46
26	KM 33	Beta-sitosterol	33.23	1.92
27	KM 35	*Grewia mollis* A.Juss. roots	67.64	24.16
28	KM 36	*Teclea simplicifolia* (Engl) Verdoorn. roots	17.93	15.03

29	KM 41	*Erythrina abssinica* D C. stem bark	29.342	21.00
30	KM 42	*Clausena anisata* (Willd) Benth. roots	9.25	10.01
31	KM 43	*Leptotrichilia* spp., leaves	53.95	30.99
32	KM 44	Coconut water extract.	14.46	6.63
33	KM 45	Coconut water extract.	13.63	5.00
34	KM 48	*Azadirachta indica* A.Juss leaves	36.23	4.17
35	KM 52	Kigelia africana (Lam.) Benth., leaves	33.07	11.13
36	KM 53	Kigelia africana, (Lam.) Benth., fruit	13.24	0.0
37	KM 56	*Prunus africana* (Hook.f.) Kalkm stem bark, MeOH extract	100.11	97.19
38	KM 57	*Prunus africana* (Hook.f.) Kalkm stem bark	29.27	22.98
39	KM 58	*Prunus africana* (Hook.f.) Kalkm stem bark, ethanol extract	99.55	78.38
40	KM 59	*Prunus africana* (Hook.f.) Kalkm stem bark, chloroform extract	99.72	85.01
41	KM 60	*Azadirachta indica* A.Juss stem bark,	96.95	89.01
42	KM 61	Fraction-1 from chromatography of KM 56	11.81	11.00
43	KM 62	Fraction-2 from chromatography of KM 56	54.28	14.37
44	KM 63	Fraction-3 from chromatography of KM 56	80.17	42.30
45	KM 64	Fraction-4 from chromatography of KM 56	98.90	80.76
46	KM 65	Fraction-5 from chromatography of KM 56	97.57 99.71	91.86 98.31
47	KM 66	Fraction-6 from chromatography of KM 56	99.06	99.22
48	KM 67	Fraction-7 from chromatography of KM 56	98.90	95.00
49	KM 68	*Prunus africana* (Hook.f.) Kalkm methanol extract after successive extraction with hexane and chloroform	98.60	97.60
50	KM 69	*Prunus africana* (Hook.f.) Kalkm ethanol extract	99.55	78.38
51	KM 70	*Prunus africana* (Hook.f.) Kalkm chloroform extract	99.72	85.01
52	KM 71	Kenya tea extract	84.1	48.6
53	KM 72	*Azadirachta indica* A.Juss stem bark-ethanol extract	99.18	88.15
54	KM 73	*Acacia mellifera* (Vahl) Benth stem bark	30.48	25.38
55	KM 78	*Prunus africana* (Hook.f.) Kalkm bark, sephadex LH-20 high molecular weight chromatographic. fraction	93.30	54.12

Table 2: Inhibition of HIV-1 Reverse Transcriptase by medicinal plant extracts – Summary of the most active plant extracts.

Ser. No.	Code No.	Plant Family	Plant name & Part used	100 µg/ml Inhibition	50 µg/ml Inhibition
1	KM 07	CELASTRACEAE	*Maytenus buchnanii* (Loes) Wilczek. - roots	89.65	68.76
2	KM 08	CELASTRACEAE	*Maytenus buchananii* (Loes) Wilczek – stem bark	99.16	95.22
3	KM09	CELASTRACEAE	*Maytenus senegalensis* (Lam.) Exell. roots	99.83	94.09
4	KM 31	LEGUMINOSAE	*Acacia mellifera* (Vahl) Benth. stem bark	99.25	84.81
5	KM 19	LEGUMINOSAE	*Erythrina abyssinica*, DC, stem bark	100.17	15.89
6	KM 41	LEGUMINOSAE	*Erythrina abyssinica* DC., stem bark	21.00	29.342
7	KM 11	MELIACEAE	*Azadirachta indica* A.Juss. stem bark	96.95	89.01
8	KM 12	MELIACEAE	*Azadirachta indica* A.Juss. stem wood	92.20	81.20
9	KM 32	MELIACEAE	*Azadirachta indica* A.Juss. root bark	59.64	35.46
10	KM 72	MELIACEAE	*Azadirachta indica* A.Juss. stem bark, ethanol extract	99.18	88.15
11	KM 43	MELIACEAE	*Leptotrichilia* sp., leaves	53.95	30.99
12	KM 15	MELIACEAE	*Melia azedarach* L. leaves	99.89	45.65
13	KM 29	MYRICACEAE	*Myrica salicifolia* A. Rich. root bark	90.23	68.79
14	KM 21	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm. stem bark	99.29	71.23
15	KM 56	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm. stem bark	100.11	97.19
16	KM 61	ROSACEAE	Fraction-1 from chromatography of KM 56	11.81	11.00
17	KM 62	ROSACEAE	Fraction-2 from chromatography of KM 56	54.28	14.37
18	KM 63	ROSACEAE	Fraction-3 from chromatography of KM 56	80.17	42.30
19	KM 64	ROSACEAE	Fraction-4 from chromatography of KM 56	98.90	80.76

20	KM 65	ROSACEAE	Fraction-5 from chromatography of KM 56	99.71	98.31
21	KM 66	ROSACEAE	Fraction-6 from chromatography of KM 56	99.06	99.22
22	KM 67	ROSACEAE	Fraction-7 from chromatography of KM 56.	95.00	98.90
23	KM 68	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm. methanol extract after successive extraction with hexane and chloroform	98.60	97.60
24	KM 69	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm. ethanol extract.	99.55	78.38
25	KM 70	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm. $CHCl_3$ ext.	99.72	85.01
26	KM 78	ROSACEAE	*Prunus africana* (Hook.f.) Kalkm bark, sephadex LH-20 high molecular weight chromatographic. fraction	93.30	54.12
27	KM 73	LEGUMINOSAE	*Acacia mellifera* (Vahl) Benth stem bark	30.48	25.38
28	KM 72	MELIACEAE	*Azadirachta indica* A.Juss stem bark- ethanol extract	99.18	88.15
29	KM 35	TILIACEAE	*Grewia mollis* A.Juss. roots	67.64	24.16

Table 3: Preliminary phytochemical screening of methanol extract from *Prunus africana* stem bark

Extracts/Fractions	Phenolic compounds	Alkaloids	Terpenoids
KM56	+++	-	++
KM61	-	-	+++
KM62	+	-	+
KM63	++	-	+
KM64	+++	-	-
KM65	+++	-	-
KM66	+++	-	-
KM67	+++	-	-

Preliminary phytochemical screening. +++ = In abundance; ++ = In moderate amounts; + = low quantities; - = none detected.

Discussion

The report presented here has shown the use of a screening method for anti-retroviral compounds by the application of HIV-1 reverse transcriptase assay. Total fractions from a carefully selected number of African (Kenyan) medicinal plants were investigated. The use of ethnopharmacoepidemiological concepts enabled the selection of fewer plants than would have been possible in such a screening.

The 10 medicinal plants which yielded the most promising extracts by this screening procedure are among some of the common medicinal plants used in African traditional medicine. The potential anti-retroviral components in these plants, therefore pose such scientific and medico-social challenge, that these plants should be investigated vigorously to enable their application in the clinic. It is significant to mention that none of these plants has been claimed by any traditional healer to be used in the preparation of medicines for AIDS. But this is not surprising since such information is always the healer's secret.

The results obtained so far seem to indicate that the enzyme inhibitory compounds are polyphenolic in nature as exemplified from the chemical and chromatographic fractionation of extracts from *Prunus africana* stem bark. Yet, there seems to be a great deal of selectivity among the extracts. Indeed, in a parallel study report [12] where these extracts were screened for activity against herpes simplex virus, most of the extracts inhibiting the HIV-1 enzyme also showed activity against the HSV. Thus, although such observations are not conclusive evidence, they tend to indicate that the extracts from these medicinal plants may contain substances of wide ranging activity which would require further investigation.

Conclusion

The reverse transcriptase inhibitory assay as reported in this study appears to be a good biological model for screening extracts and compounds from plants for anti-HIV activity. It would be desirable to use this model to isolate pure compounds as potential anti-retroviral agents. These compounds will serve as reference substances for the standardization of the total extracts which can be investigated as herbal remedies in the treatment of AIDS in humans. The advantage of this approach is that there is a substantial background on safety from ethnopharmacoepidemiological information which can be used in the preliminary investigations on the safety and efficacy of such preparations.

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