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Inhibitory effects of some Panamanian plants on human immunodeficiency viral reverse transcriptase and protease

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Abstract

In the course of our studies on the development of anti-HIV agents, we have screened thirty-nine Panamanian plants for their inhibitory activity against HIV-1 reverse transcriptase (RT) and protease (PR), essential enzymes for proliferation of HIV. Water extracts of *Chamaesyce hyssopifolia* (whole plant), *Cordia spinescens* (leaves), and *Hyptis lantanifolia* (aerial parts), and the methanol extracts of *Tetrapteris macrocarpa* (aerial parts) and *Xylopia frutescens* (leaves) appreciably inhibited the activity of HIV-1 RT, with IC_{50} of 8, 6, 7, 8, and 11 μg/ml, respectively. Furthermore, the methanol extracts of *Erythroxylum citrifolium* (trunk), *Serjania mexicana* (whole plant), *Waltheria indica* (branches), and a water extract of *Lindackeria laurina* (leaves) showed appreciable inhibition against HIV-protease, with IC_{50} of 58, 48, 46, and 54 μg/ml, respectively.

Key words AIDS, human immunodeficiency virus, Panamanian plants, protease, reverse transcriptase.

Abbreviations AIDS, acquired immunodeficiency syndrome ; AZT, azidothymidine ; DDC, dideoxycytidine ; DDI, dideoxyinosine ; $(dT)_{12-18}$, oligo deoxythymidylic acid ; HIV, human immunodeficiency virus ; HPLC, high performance liquid chromatography ; IC_{50} 50 % inhibitory concentration ; $(rA)_n$, poly riboadenylic acid ; PR, protease ; RT, reverse transcriptase.

Introduction

The development of potent antiviral drugs to arrest the infection by human immunodeficiency virus (HIV), a causative agent of acquired immunodeficiency syndrome (AIDS), still remains an urgent need. An effective chemotherapy for AIDS treatment may be the use of a combination of drugs that can act at different steps in the life cycle of HIV. Potential targets for the inhibition of HIV replication include two virally encoded enzymes : reverse transcriptase (RT)[1] and protease (PR).[2,3] Reverse transcriptase has a key role in the early stage of HIV infection, which is responsible for converting the viral genomic RNA into proviral double-stranded DNA. Protease is an aspartic protease that processes viral polyproteins to produce mature and infectious viral particles. Therefore, both are key target enzymes for the inhibition of viral replication. The HIV-1 RT inhibitors approved for clinical use are the nucleoside analogs AZT, DDI, DDC and recently, D4T (2′, 3′-didehydro-3′-deoxy-thymidine).[4] However, their uses have been limited by the rapid emergence of resistant-strains and the toxicity associated to their inhibition of the host DNA polymerases. Protease inhibitors reported so far are peptide-derived drugs, however, most of them have limited use due to low oral bioavailability

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and rapid excretion.[5] Recently, protease inhibitors have begun to be used clinically in combination with nucleoside analogues, but are more recommended for high risk exposures and for persons who showed resistance to the RT inhibitors.[6] Taking into account that herbal medicines are popularly recognized for their easy preparation, administration, safety and low cost, they would be the most reasonable materials to search for an effective drug, from a pharmaceutical and economical point of view.

For the purpose of finding specific and potent inhibitors of HIV, we have screened various traditional medicines for their inhibitory effects on the infection of HIV-1 in cultured cells[7] and on reverse transcriptase and protease.[8-14] At present, we report on the inhibitory effects of some Panamanian medicinal plants on the activity of HIV-1 RT and PR.

Materials and Methods

Plant materials : Plants were collected in the Republic of Panama by botanists of the Pharmacognosy Research Unit under the program FLORPAN of the University of Panama, and identified by Mrs. Carmen Galdames, Alex Espinosa and Eduardo Valdes. The voucher specimens were deposited at the herbarium of the University of Panama.

Preparation of extracts : Ten grams of a dried plant sample were extracted with 200 ml of methanol or water by refluxing for 3 h. After filtration, the filtrate was concentrated under reduced pressure and then dried.

Enzymes and chemicals : Recombinant HIV-1 RT was purchased from Eiken Chemicals Co. Ltd. (Osaka, Japan). A template-primer, $(rA)_n \cdot (dT)_{12-18}$ was obtained from Pharmacia (Uppsala, Sweden). [methyl-^{3}H]-Thymidine 5′-triphosphate (dTTP) (specific activity, 1.70 TBq/mmol) was obtained from Amersham-Japan (Tokyo, Japan) and the scintillation fluid Aquasol-2 from NEN Research Products (Boston, USA). Adriamycin (doxorubicin hydrochloride) was purchased from Sigma Chemical Co. (St. Louis, USA). Recombinant HIV-1 PR was prepared according to the method described by Kusumoto *et al.*[12] Purification of the protein containing HIV-1 protease was achieved by HPLC (reversed-phase, Vydac C_4, 0.46×25 cm, solvent system : 30 % 2-propanol/70 % acetonitrile in 0.1 % trifluoroacetic acid, at a flow rate of 0.9 ml/min and detected at 280 nm. The undecapeptide, His-Lys-Ala-Arg-Val-Leu-(pNO_2-Phe)-Glu-Ala-NLe-Ser-NH_2 used as a substrate corresponding to the p24-p15 cleavage site of HIV-1 protease, was obtained from Peptide Institute, Inc. (Osaka, Japan). A stock solution of the peptide (0.2 mg/ml) was prepared in 50 mM NaOAc (pH 4.96).

Reverse transcriptase assay : This assay was performed according to the Method described by El-Mekkawy.[14]

Protease assay : A reaction mixture (5 μL) containing 50 mM acetate buffer, pH 5.0, 1.5 mM of substrate, 1 μL of a plant extract and 3.4 μM HIV-1 PR solution were added and the mixture was incubated at 37°C for 120 min. The extracts tested were dissolved in distilled water or dimethyl sulfoxide (DMSO, 10 % in the reaction mixture). A control reaction was performed under the same conditions, without the addition of plant extracts. The reaction was stopped by heating the mixture at 90°C for 1 min. Then, 35 μL of water were added and an aliquot of 5 μL was analyzed by HPLC. Acetylpepstatin (Bachem Feinchemikalien AG, Bubendort, Switzerland) which showed 50 % inhibitory activity (IC_{50}) at 29 μg/ml, was used as positive control of inhibition.

HPLC : The HPLC system consisted of an LC9A liquid chromatograph, SPD-6A UV spectrophotometric detector, SLC-6B autoinjector and an integrator C-R6A Chromatopac (Shimazu Corporation, Kyoto, Japan) were used. Five microliters of the reaction mixture were injected into a RP-18 column (4.6×150 mm, Merck, Darmstadt, Germany), eluted with a gradient of acetonitrile (20-40 %) in 0.1 % trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The substrate and Phe-pNO_2-bearing hydrolysate were eluted at 9.44 and 4.35 min, respectively. The PR activity was calculated from the ratio of the substrate peak area to the product peak area.

Results and Discussion

As part of our work on naturally occurring antiviral agents, we investigated the HIV-1 RT and

PR inhibitory effects of some Panamanian plant extracts. These plants are commonly used for the treatment of several ailments such as infectious wounds, inflammation, fevers, skin diseases and gastrointestinal disorders, and in the present experiment, it was found that some of them had potential inhibitory effects against HIV-1 RT and PR. Results are shown in Table I as the concentration that inhibits 50 % of the enzyme activity, IC_{50}.

Five extracts showed strong inhibitory effects

Table I HIV-1 reverse transcriptase and protease inhibitory activity of Panamanian plant extracts

Botanical name	Family name	Parts	Extract	IC_{50} (μg/ml) vs.RT	IC_{50} (μg/ml) vs.PR
Acalypha macrostachya Jacq.	Euphorbiaceae	branches	water	500	>1000
			methanol	1000	1000
		leaves	water	173	1000
			methanol	>1000	>1000
Aegiphila anomala Pitt.	Verbenaceae	leaves	water	24	87
Aphelandra sinclairiana Nees	Acanthaceae	branches	water	>1000	>1000
			methanol	>1000	>1000
		leaves	water	>1000	>1000
			methanol	1000	>1000
Baccharis pedunculata (Mill.) Cabr.	Compositae	aerial part	water	55	1000
Baccharis trinervis (Lam.) Pers.	Compositae	aerial part	water	50	>1000
Begonia glabra Aubl.	Begoniaceae	whole plant	water	110	1000
Bidens pilosa L.	Compositae	aerial part	water	325	1000
Bursera simaruba (L.) Sarg.	Burseraceae	trunk	methanol	>1000	>1000
Calea jamaicensis (L.) L.	Compositae	branches	water	15	1000
		roots	water	56	>1000
			methanol	>1000	>1000
Cephaelis camponutans Dwyer & Hayden	Rubiaceae	aerial part	methanol	46	>1000
Chamaesyce hyssopifolia (L.) Small	Euphorbiaceae	whole plant	water	8	1000
			methanol	43	1000
Commelina diffusa Burm.f.	Commelinaceae	whole plant	water	233	>1000
Cornutia grandifolia (Schlecht. & Cham.) Schau.	Verbenaceae	trunk	water	450	100
Cordia spinescens L.	Boraginaceae	leaves	water	6	100
			methanol	36	>1000
Croton billbergianus Muell. -Arg.	Euphorbiaceae	trunk	water	>1000	>1000
Drymonia serrulata (Jacq.) Mart.	Gesneriaceae	leaves	water	193	1000
Erythroxylum citrifolium St. Hil.	Erythroxylaceae	trunk	methanol	50	43
E.lucidum H.B.K.		leaves	methanol	47	1000
Faramea eurycarpa J.D.Sm.	Rubiaceae	leaves	water	>1000	>1000
		roots	water	>1000	>1000
Guazuma ulmifolia Lam.	Sterculiaceae	leaves	water	450	1000
			methanol	40	1000
Hamelia axillaris Swartz	Rubiaceae	branches	water	>1000	>1000
			methanol	400	400
		leaves	water	1000	1000
			methanol	666	1000
Hoffmannia woodsonii Standl.	Rubiaceae	leaves	methanol	>1000	>1000
Hyptis brevipes Poit.	Labiatae	aerial part	water	75	>1000
H. capitata Jacq.		aerial part	methanol	>1000	>1000
H. lantanifolia Poit.		aerial part	water	7	100
H. obtussiflora Presl.		aerial part	methanol	100	1000
Jatropha curcas L.	Euphorbiaceae	branches	water	100	>1000
			methanol	730	>1000
		leaves	water	50	1000
			methanol	129	1000

Table I, continued

Lindackeria laurina Presl.	Flacourtiaceae	leaves	water	193	54
			methanol	19	1000
Mikania banisteriae DC.	Compositae	branches	water	44	1000
			methanol	>1000	>1000
		leaves & flowers	water	35	100
			methanol	1000	1000
Pavonia schiedeana Steud.	Malvaceae	aerial part	methanol	16	1000
Peltastes colombianus Woods.	Apocynaceae	branches	water	>1000	>1000
			methanol	>1000	1000
		leaves	water	700	>1000
			methanol	1000	1000
Pereskia bleo (H.B.K.) DC.	Cactaceae	whole plant	water	>1000	>1000
Polygonum punctatum Ell.	Polygonaceae	roots	methanol	100	1000
Psychotria camponutans Dwyer & Hayden	Rubiaceae	aerial part	methanol	46	>1000
Rauvolfia littoralis Rusby	Apocynaceae	leaves & branches	methanol	>1000	>1000
Ruellia biolleyi Lindau.	Acanthaceae	whole plant	methanol	1000	>1000
Serjania mexicana (L.) Willd.	Sapindaceae	whole plant	water	55	87
			methanol	35	1000
Tetrapteris macrocarpa Johnst.	Malpighiaceae	aerial part	methanol	8	>1000
Waltheria indica L.	Sterculiaceae	branches	water	70	96
			methanol	50	48
		leaves	water	425	1000
			methanol	400	145
Xylopia frutescens Aubl.	Annonaceae	leaves	methanol	11	415
		bark	methanol	22	46

against HIV-1 RT : the water extracts of *Chamaesyce hyssopifolia* (whole plant), *Cordia spinescens* (leaves), *Hyptis lantanifolia* (aerial part), and the methanol extracts of *Tetrapteris macrocarpa* (aerial part) and *Xylopia frutescens* (leaves) with IC_{50} of 8, 6, 7, 8 and 11 μg/ml, respectively. The water extracts of *Aegiphila anomala* (leaves), *Baccharis trinervis* (aerial part), *Calea jamaicensis* (branches and roots), *Jatropha curcas* (leaves), *Mikania banisteriae* (leaves and branches) and methanol extracts of *Psychotria camponutans* (aerial part), *Erythroxylum citrifolium* (trunk), *E. lucidum* (leaves), *Guazuma ulmifolia* (leaves), *Lindackeria laurina* (leaves), *Pavonia schiedeana* (aerial part), *Xylopia frutescens* (bark) and *Waltheria indica* (branches) also showed appreciable inhibitory activity, IC_{50} = 19-50 μg/ml.

Regarding the PR inhibition, the methanol extracts of *Erythroxylum citrifolium* (trunk), *Waltheria indica* (branches), *Xylopia frutescens* (bark), and water extracts of *Lindackeria laurina* (leaves) showed relatively strong inhibition with IC_{50} of 58, 48, 46, and 54 μg/ml, respectively. Some other extracts that showed moderate inhibition were the water extracts of *Aegiphila anomala* (leaves), *Cornutia grandifolia* (trunk), *Cordia spinescens* (leaves), *Hyptis lantanifolia* (aerial parts), *Mikania banisteriae* (leaves and flowers), *Serjania mexicana* (whole plant) and *Waltheria indica* (branches), with IC_{50} = 87-100 μg/ml.

Concerning the chemical constituents present in related species among most active plant extracts, such as those of *C. hyssopifolia,* flavonoids[15] and hydrolizable tannins[16] are reported, and have shown activity against RT[8, 10, 11, 17, 18] and HIV replication in cell culture,[18] while highly polyphenolic lignans were isolated from *Cordia goetzei,*[19] oxoaporphine alkaloids[20] and acetogenins[21] from *Xylopia* spp. Moreover, it has been reported the anti HIV activity of hot water and alkaline extracts of another *Erythroxylum* sp.,[22] which are well-known to contain alkaloids belonging to tropanes and pyrrolidines.[23] To determine whether or not, these types of substances are responsible for the activity found in the present experiment, a more detailed investigation is now in progress.

和文抄録

抗エイズウイルス薬の開発を目指し，エイズウイルス(HIV)の増殖に必須な酵素である逆転写酵素(RT)，プロテアーゼ(PR)を指標に39種のパナマ植物エキスの阻害作用を検討した。*Chamaesyce hyssopifolia*（全草），*Cordia spinescens*（葉），*Hyptis lantanifolia*（地上部）の水エキス，*Tetrapteris macrocarpa*(地上部)，*Xylopia frutescens*(葉)のメタノールエキスはHIV-1 RTを顕著に阻害した。それぞれの50％阻害濃度は8, 6, 7, 8および11 μg/mlであった。さらに，*Erythroxylum citrifolium*(樹幹)，*Serjania mexicana*（全草），*Waltheria indica*(枝)の水エキスおよび*Lindackeria laurina*（葉）のメタノールエキスはHIV-PRを阻害し，50％阻害濃度は58, 48, 46, 54 μg/mlであった。以上の結果はこれら生薬（その含有成分も含む）が抗エイズウイルス薬開発の候補となり得ることを示している。

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