Screening of South American Plants against Human Immunodeficiency Virus: Preliminary Fractionation of Aqueous Extract from Baccharis trinervis

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Ethanolic and aqueous extracts of 14 South American medicinal plants were tested for inhibitory activity on human immunodeficiency virus (HIV). Both extracts were relatively non-toxic to human lymphocytic MT-2 cells, but only the aqueous extract of Baccharis trinervis exhibited potent anti-HIV activity in an in vitro MTT assay. To delineate the extract-sensitive phase, some studies of the antiviral properties of the active extract are described in this paper. Based on the results presented here, a separation scheme was devised, which permitted the preliminary fractionation of the extract, with the aim of finding an inhibitor of this virus.

Key words South American medicinal plant; antiviral activity; Baccharis trinervis; HIV; AIDS

The control of viral diseases has been the subject of intense scientific endeavour, with special attention being devoted to those having retroviruses as etiological agents, including acquired immunodeficiency syndrome (AIDS). AIDS is a pandemic immunosuppressive disease which results in life-threatening opportunistic infections and malignancies. Since a retrovirus, designated human immunodeficiency virus (HIV), has been clearly identified as the primary cause of this disease,1,2 numerous compounds have been evaluated for their inhibitory effects on HIV replication in vitro. HIV has two main targets in vivo: CD4 lymphocytes and tissue macrophages. Treatments aimed at the control of HIV replication in both cell types are envisaged.

According to De Clercq,3 the replicative cycle of HIV comprises ten steps that could be considered suitable targets for chemotherapeutic intervention. A number of laboratories are actively involved in the development of antiviral agents that interfere with HIV at different stages of viral replication.4,5 Most of the anti-HIV compounds can be assigned to one of these ten classes of HIV inhibitors, according to the stage at which they interfere with the HIV replication cycle, e.g., adsorption, fusion, uncoating, reverse transcription, integration, DNA replication, transcription, translation, maturation and budding (assembly/release). However, only three groups of drugs (nucleoside and non-nucleoside analogs of reverse transcriptase and protease inhibitors) have been approved for general application, although prolonged use of these agents is limited because of their toxicity and the development of drug resistance. The high mutation rate of HIV frequently results in the rapid development of resistance towards the drugs used,6 and an attempt has been made to circumvent this problem by using a combination of drugs. AIDS therapy may require the use of a combination of agents that exhibit synergistic antiviral effects to prevent the emergence of drug-resistant HIV mutants. Combination of three or more drugs reduces the viral load to undetectable levels and results in the prolonged survival of treated patients.

A phytotherapeutic approach to modern drug development can provide many invaluable drugs from traditional medicinal plants. Over the last decade, antiviral researchers have also turned to many traditional folk medicines, invariably a “cocktail” of natural products, to uncover the scientific basis of their remedial effects. Many plant products are being used by patients with AIDS in some countries without any scientific proof that they possess anti-HIV activity. Traditional healers are now offering their remedies for scientific evaluation, and a number of studies provide information on the inhibitory activity against HIV of selected plants.7–9 A large number of plant-derived substances have been described that exhibit anti-HIV activity, e.g., alkaloids, polysaccharides, lignans, flavonoids, coumarins and terpenes.10–12 Some of the compounds that have been reported to inhibit HIV replication cannot be unequivocally allocated to one of the ten classes of HIV inhibitors, primarily because their target of action has not been elucidated.

In our search for new classes of antiviral agents, we have examined extracts of several South American medicinal plants, some of them used in traditional medicine. The results presented here concern several Ecuadorian and Bolivian medicinal plants from the following species: Baccharis trinervis Pers., Baccharis teindalensis L., Baccharis genistelloides (Lam.) Pers., Baccharis rubricaulis Rusby, Ambrosia arborescens Miller, Eupatorium articulatum Hort., Eupatorium glutinosum Lam., Tagetes pusilla H.B. & K., Neurolaena lobata R. Br. and Conyza floribunda H.B. & K. from the Compositae family; Phoradendron crassifolium (Polil.) Eicher from Loranthaceae; Rumex obtusifolius L. from Polygonaceae; Plantago australis Lam. from Plantaginaceae, and Satureja boliviana (Benth.) Briquet from the Lamiaceae family.

Early studies in our preliminary screening of those extracts showed that some of them presented antiviral activity against herpes simplex type I (HSV-1) and vesicular stomatitis virus (VSV).13,14 Thus, aqueous extracts of E. glutinosum, T. pusilla, A. arborescens, R. obtusifolius, P. australis and B. teindalensis inhibited VSV replication, while P. crassifolium...
and B. trinervis exhibited antiviral activity against HSV-1. Three of these plants, namely E. articulatum, B. genistelloides and S. boliviana exhibited antiviral effects against both DNA and RNA viruses (HSV-1 and VSV, respectively), which are unrelated from a phylogenetic viewpoint.

In view of these results, it was decided to further investigate these plants for their antiviral activity against HIV, with the aim of finding an inhibitor of this virus. Based on the results presented here, a separation scheme was devised which permitted the preliminary fractionation of the active extract. The separation procedure and some studies of the antiviral properties of the active extract are described in this paper.

MATERIALS AND METHODS

Plant Material and Extracts  B. trinervis, B. teindalensis, E. articulatum, E. glutinosum, T. pusilla, N. lobata and C. floribunda were collected in the flowering season from different geographical locations of Ecuador. Two extracts, Ethanolic and aqueous extracts, were prepared from each plant species, and were provided by the School of Biochemistry and Pharmacy, Central University of Quito, Ecuador. B. genistelloides, B. rubricaulis, A. arborescens, P. crassifolium, R. obtusifolius, P. australis and S. boliviana were collected in the flowering season from different regions of the north and center of La Paz, Bolivia. The above mentioned extracts were prepared from each plant species, and were provided by the Faculty of Pharmacy, Mayor University of San Andres, La Paz, Bolivia.

Cells and Virus  MT-2 cells15) were cultured in RPMI 1640 medium (Whitaker M.A. Bio-products, Walkerville, MD, U.S.A.) containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml). MT-2 cells were cultured at 37 °C in a 5% CO2 humidified atmosphere and splinted twice a week. Macrophages were obtained from the peripheral blood of healthy seronegative donors (PBMC) using centrifugation on a Ficoll–Hyphaque gradient and plate adherence. The mononuclear cells obtained through Ficoll–Hyphaque were seeded in 24-well plates at a concentration of 2×10⁵/ml of complete medium (10% fetal calf serum, 10% human AB serum in RPMI 1640 supplemented with L-glutamine and penicillin–streptomycin). Nonadherent cells were then removed by repeated gentle washing with warm medium. Adherent cells obtained by this procedure are more than 95% pure for the CD14 monocyte marker. Cultures were maintained in complete medium for the whole experiment. MT-2 cells and macrophages were infected with HIV-1 NL 4.3 (X4 strain) or BaL (R5 strain), respectively.16,17) NL 4.3 was obtained from the culture supernatant of MT-2 cells infected with this virus, as described by Richman.18) Virus stock was titrated by endpoint dilution of the cytopathic effect in MT-2 cells, and p24 antigen concentrations in the supernatant were measured by ELISA. BaL was obtained from the supernatant of PBMCs infected with this virus, and p24 antigen concentrations in the supernatant were measured by ELISA, according to the manufacturer’s instructions.

Antiviral Assay  The anti-HIV activity and toxicities of extracts were assessed in MT-2 infected with NL 4.3. MT-2 cells uninfected or HIV-infected (20 ng/well for p24 antigen at a density of 2×10⁵ cells/well in 100 μl of medium) were seeded in 96-well flat-bottomed microtiter culture plates with 100 μl of different concentrations of the test samples. After 7 d of incubation at 37 °C, cell viability was determined by MTT assay.19) Briefly, 20 μl of an MTT stock solution (7.5 mg/ml in phosphate buffered saline (PBS)) was added to each well. After 1 h of incubation at 37 °C, 150 μl of the medium was carefully removed without disturbing cells containing formazan crystals. Solubilization of the formazan crystals was achieved by adding 100 μl 10% (v/v) Triton X-100 in acidified isopropanol (0.04 m HCl in isopropanol). Finally, the absorbance was read in a computer-controlled photometer. The absorbance measured at 690 nm was automatically subtracted from the absorbance at 540 nm to eliminate the effects of non-specific absorption.

Fractionation of Active Extract  A preliminary bioassay-guided fractionation of the active extract (the aqueous extract of aerial parts from B. trinervis) was performed. An aqueous solution of the extract was very viscous, presumably due to the presence of mucilages and polysaccharides. The crude aqueous extract was dissolved in ethanol to remove organic molecules. The remaining water-soluble extract was first passed through a Sephadex LH-20 column (23 × 1.2 cm) in water as a preliminary fractionation. Four fractions (1—4) were obtained. The above-mentioned antiviral test was used to monitor the purification.

Anti-HIV Activity in Macrophages of the Active Extract  The assay of anti-HIV activity in macrophages was performed 5 d after the separation. Macrophages (2×10⁵ cells) were infected with BaL (100 ng/well of p24 antigen) in 1 ml of medium with or without an aqueous extract of B. trinervis (400 μg/ml). Cells were exposed to the virus overnight, then the medium was completely removed and washed twice with PBS. Macrophages uninfected or infected were cultured in the absence or presence of an active extract in 1 ml of medium for 13 d, and p24 antigen concentrations in the supernatant were measured by ELISA.

RESULTS AND DISCUSSION

Two extracts each of 14 South American medicinal plants were examined for their ability to inhibit HIV replication. Both ethanolic and aqueous extracts were relatively nontoxic to human lymphocytic MT-2 cells, but most of them had no antiviral effect; only the aqueous extract of B. trinervis exhibited potent anti-HIV activity in an in vitro MTT-based assay. The results presented here indicated that this extract possesses anti-HIV properties of therapeutic interest. This extract is active against the two major targets of HIV: lymphocytes and macrophages. The extract exhibited antiviral activity against HIV in MT-2-infected cells at a concentration ranging from 10 to 400 μg/ml (Fig. 1). No cytotoxic effects were observed even at the maximum concentration tested (400 μg/ml). The selectivity index (cytotoxic concentration₅₀/inhibitory concentration₅₀) was 4882.

To delineate the extract-sensitive phase, time-of-addition experiments were carried out. In these experiments, macrophages were infected with HIV at a high titer, and active extract was added at different times during viral infections. The extract inhibited HIV replication under conditions in which it was added at the same time as the viral infection. On the other hand, when extract was added only during viral
adsorption, a slight decrease in the kinetics of HIV replication was observed, although the active extract inhibited the virus replication even after penetration of the virus into the cells. Differences observed when the extract was added only after adsorption or was maintained thereafter were not significant, and detectable p24 levels were very close to the threshold of the technique (Fig. 2). Thus, these findings indicate that the main target for antiviral activity of an aqueous extract of \textit{B. trinervis} could be estimated to be the early steps of virus replication, including virus-cell attachment, virus-cell fusion and cell-to-cell fusion.

The fractionation of pharmacologically active compounds from the aqueous extract of \textit{B. trinervis} was monitored by tests on the above mentioned MTT-antiviral assay. When distributed between ethanol and water, the aqueous phase was found to contain compounds with anti-HIV activity, whereas the ethanolic phase was inactive (data not shown). A water-soluble fraction from the extract was first purified on a Sephadex LH-20 column. Four fractions (1—4) were obtained. The active fractions (1 and 2) were the first to be eluted. The percentage yields were (W/W crude aqueous extract): 0.68 mg% for fraction 1 and 3.42 mg% for fraction 2. Both fractions inhibited HIV replication in MT-2-infected cells, without showing cytotoxic effects, at concentrations ranging from 10 to 200 μg/ml (Fig. 3A and 3B). The selectivity index was 2550 for fraction 1 and 22135 for fraction 2.

In a preliminary chemical characterization, the active compounds appeared to be of high relative molecular weight, probably due to the presence of polysaccharides. This type of compound has been reported in the literature as a potent inhibitor of different enveloped viruses, including HIV.\cite{20-24} These purified fractions were evaluated in the antiviral assay, but were not further analyzed chemically.

Our studies indicate that the aqueous extract of \textit{B. trinervis} contains antiviral activity that may be useful in the treatment of patients with AIDS. Further studies are needed to determine the chemical identification of the active constituents in \textit{B. trinervis} and their potential mechanisms of action.

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