In Vitro Anti-Human Immunodeficiency Virus Activity of Polysaccharide from Rhizophora mucronata Poir.

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A polysaccharide was extracted with 1% sodium carbonate from the bark of Rhizophora mucronata and its antiviral activities against human immunodeficiency virus (HIV) were assessed by an in vitro cell culture system. The anti-HIV activity of the alkaline extract was mainly recovered in the 25–75% ethanol-precipitated fraction. Rhizophora mucronata polysaccharide (RMP) protected MT-4 cells from the HIV-induced cytopathogenicity and blocked the expression of HIV antigens. RMP completely inhibited the viral binding to the cell and the formation of syncytium upon cocultivation of MOLT-4/HIV-1INB cells and MOLT-4 cells. These results suggest that RMP inhibited early steps of the virus life cycle especially virus adsorption to the cell.

Key words: Rhizophora mucronata; polysaccharide; human immunodeficiency virus; anti-HIV activity; indirect immunofluorescence assay

Infection with the human immunodeficiency virus (HIV) and its sequel, acquired immunodeficiency syndrome (AIDS), remain a health threat of global significance. Because of the limitations of currently available therapies, an extensive search for new anti-HIV agents is ongoing. The existing therapies have targeted reverse transcriptase and protease enzyme activities as a method of inhibiting viral replication. The development of drug-resistant strains1,2 and the occurrence of side-effects3–5 are major disadvantages of the present therapies for the treatment of AIDS. It is essential to find indigenous drugs for the treatment of HIV infection as HIV infection is rapidly spreading in India and currently available anti-HIV drugs are very expensive and limited in supply.

Rhizophora mucronata Poir., a mangrove plant belonging to the family Rhizophoraceae has been used in folklore medicine in India.6 The plant has been shown to have a broad-spectrum antiviral activity.7 We report here that an alkaline extract from the bark of R. mucronata has strong inhibitory effects on HIV replication and HIV-induced cytopathogenicity in MT-4 cells and on HIV-induced syncytium formation in a coculture of MOLT-4/HIV-1INB cells and MOLT-4 cells. The substance responsible for the anti-HIV activity of the plant extract is an acid polysaccharide.

Materials and Methods

Reagents and chemicals. The following reagents were obtained from the indicated companies: RPMI 1640 medium (Gibco, Grand Island, NY); fetal calf serum (FCS) (Whittaker Bioproduct, Walkersville, MD); 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemicals, Osaka, Japan); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Cappel Organon Teknika Co., West Chester, PA).

Preparation of plant powder. Bark of Rhizophora mucronata was collected from Pichavaram mangrove forest (11°27'N; 79°47'E), Tamil Nadu, India. The specimen was identified and its holotype has been deposited in the herbarium of the centre of Advanced Study in Marine Biology, Parangipettai, Tamil Nadu, India. The samples were washed, shade-dried, and powdered.

Different solvent extraction. The powdered plant sample (10 g) was extracted with 100% ethanol (500 ml) at 45°C for 3 h and then the residue was further extracted in hot water (500 ml) at 85°C for 3 h. The resulting sample residue was extracted with 1% sodium carbonate (250 ml) at 45°C for 3 h. All the solutions were evaporated and the extracts were freeze dried and tested for anti-HIV activity.

Purification of the polysaccharide from bark of Rhizophora mucronata.

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Abbreviation: RMP, Rhizophora mucronata polysaccharide
Bark of *Rhizophora mucronata*

- **hot water extraction**
  - residue
  - extract
  - **1% Na₂CO₃** extraction
    - crude extract (100)
  - crude extract dissolved in 0.1 M CH₃COONa
  - **25% ethanol precipitation**
    - precipitate (28)
    - supernatant (50% ethanol precipitation)
    - precipitate (42)
    - supernatant (75% ethanol precipitation)
    - precipitate (16)
    - supernatant (14)

**Fig. 1.** Fractional Preparation of Bark of *Rhizophora mucronata*. Number in parentheses denotes percent yield of each fraction.

**Preparation of the crude extract.** The powdered plant sample (50 g) was successively extracted twice with hot water (500 ml) at 85°C for 3 h, and twice with 1% sodium carbonate (500 ml) at 45°C for 3 h. The alkaline extract was filtered through two layers of gauze, and designated as “crude extract”.

**Ethanol precipitation.** The crude extract was fractionated in the presence of sodium acetate. The extract (100 mg) was dissolved in 0.1 M sodium acetate solution (20 ml), and 1/4 volume (6.5 ml) of 100% ethanol was added to bring the concentration of ethanol to 25% in sodium acetate solution. The mixture was left for 1 h at 4°C and then centrifuged at 150×g for 15 min. The precipitated fraction was designated as 0–25P. To the supernatant, 13.5 ml of 100% ethanol was added to bring the concentration of the ethanol to 50%, the mixture was processed as described above, and it was designated as 25–50P. The supernatant obtained was further fractionated with the addition of 40 ml of 100% ethanol to bring the concentration of the ethanol to 75% and processed as above. The final precipitated fraction and the supernatant were designated as 50–75P and 75S, respectively. The precipitated fractions between 25% and 75% ethanol were pooled and designated as 25–75P.

**Column chromatography.** The fractions from the ethanol precipitation were dissolved in distilled water, put on a column (1.6 × 50 cm) of Cellulofine GC-700m that had been previously equilibrated with distilled water, and then eluted with the same solvent.

**Analytical methods.** The contents of neutral sugars and uronic acids were estimated by the phenol-sulfuric acid method and the carbazole method, respectively. The contents of protein and sulfate were estimated as described by Bradford and Dodgson, respectively.

**Cells and viruses.** A human T lymphotropic virus type I (HTLV-I) positive T-cell line, MT-4, and a lymphoblastoid T-cell line, MOLT-4 (clone no. 8) were subcultured twice a week at a concentration of 3 × 10⁵ cells/ml in RPMI 1640 medium with 10% (V/V) heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. A strain of HIV-1₃₁₉ was prepared from the culture supernatant of MOLT-4/HTLV-1₃₁₉ cells, that were persistently infected with HIV-1₃₁₉.

**Antiviral assay by MTT method.** The inhibitory effect of the test substance on HIV replication was monitored by the inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, MT-4 cells were suspended at 3 × 10⁵ cells/ml and infected with HIV at a multiplicity of infection (m.o.i.) of 0.01. The HIV-infected or mock-infected MT-4 cells were placed in 96-well microtiter plates (200 µl/well) and incubated at 37°C in a CO₂ incubator in the presence of the test substance. After 5 days, cell viability was measured by the MTT assay, as described previously, from which 50% cytotoxic concentration (CC₅₀), 50% effective concentration (EC₅₀), and selectivity indices (SI = CC₅₀/EC₅₀) were calculated.

**Assay for viral antigen expression.** The anti-HIV efficacy was also assessed from the inhibitory effect on virus-specific antigen expression as measured by the indirect immunofluorescence method. MT-4 cells were exposed to virus for 90 min for adsorption. After adsorption, the unbound virus was removed by repeated washing with the medium and then resuspended in media containing various concentrations of the test substance and cultured at 37°C in a CO₂ incubator. After 5 days of incubation, the number of viable cells was monitored by the trypan blue dye exclusion method and the HIV-1 antigen-positive cells were monitored by indirect
immunofluorescence using a high-titer polyclonal antibody obtained from a patient with AIDS related complex, and FITC-conjugated rabbit anti-human IgG antibody.

**Syncytium formation assay.** MOLT-4 cells (5 x 10⁴) were cultured with an equal number of MOLT-4/HIV-1G12 cells in the microtiter plate wells containing various concentrations of the test substance. At 20 hours of cocultivation, the number of giant cells (syncytium) was counted and the percent inhibition of syncytium formation was calculated as follows:

\[
\text{% inhibition of syncytium} = \left(1 - \frac{\text{syncytia formed in the presence of drug}}{\text{syncytia formed in the absence of drug}}\right) \times 100
\]

The 50% inhibitory concentration was estimated from graphs of percent inhibition plotted against drug concentration.

**Virus binding assay.** The inhibitory effect of the test substance on virus binding to CD4-positive cells was measured by an indirect immunofluorescence-laser flow cytometric method.¹³,¹⁵ MT-4 cells were exposed to highly concentrated HIV-1 virions in the presence or absence of the test substance. The test substance was added 1 min before the virus was added. The cells were incubated for 60 min at 37°C and washed twice in phosphate-buffered saline (PBS) to remove the unbound virus particles. Then a high-titer polyclonal antibody obtained from a patient with AIDS related complex (diluted 1/500 in PBS) was added. After 60 min of incubation at 37°C, the cells were washed twice with PBS. The cells were then incubated with FITC-conjugated F(ab')₂ fragments of rabbit anti-human immunoglobulin antibody (diluted 1/30 in PBS) for 60 min at 37°C, washed twice in PBS, resuspended in one ml of 0.5% paraformaldehyde in PBS, and analyzed by FACScan (Becton Dickinson, Sunnyvale, CA).

**Results and Discussion**

The anti-HIV activity of different solvent extracts from the bark of *Rhizophora mucronata* is shown in Table 1. The ethanol and water extracts did not have anti-HIV activity but the alkaline extract showed the anti-HIV activity. This alkaline extract was further fractionated with ethanol in the presence of sodium acetate as described in Fig. 1. With a lower concentration of sodium acetate (0.05 M), an extremely low amount of polysaccharide was obtained between 25% and 50% ethanol, and with a higher concentration of sodium acetate (0.5 M), a high amount of polysaccharide was obtained between 0% and 25% ethanol. Therefore, we used 0.1 M sodium acetate in which the precipitation was moderate. The degree of precipitation by ethanol was also dependent on the concentration of the alkaline extract. The total recovery of the polysaccharide by ethanol precipitation was 50% to 60%. As shown in Table 2, the fraction 50-75P showed the highest activity with a 50% effective concentration (EC₅₀) value of 10.23 μg/ml. In the case of 25-50P fraction the EC₅₀ was 16.99 μg/ml. The EC₅₀ value for the 25-75P fraction was 14.12 μg/ml. However, the 75S fraction did not show anti-HIV activity.

The active fraction (25-75P) was analyzed by chromatographic profile in a column of Cellulofine GC-700m. There was a peak in fractions 9-15 (Fig. 2). These fractions were combined and designated as RMP (*R. mucronata* polysaccharide). RMP contained a large amount of neutral sugars and uronic acids (Fig. 2). Since no protein and sulfate could be detected, the polysaccharides have to be acid polysaccharides. Sulfated polysaccharides from natural resources¹⁶,¹⁷ or synthetic¹⁸,¹⁹ have been reported to have anti-HIV activity.

The anti-HIV activity of RMP was measured by the protection of HIV-induced cytopathic effect and the inhibition of HIV-specific antigen expression in MT-4 cells (Fig. 3). It showed concentration-dependent inhibition of HIV with the EC₅₀ value of 4.38 μg/ml. A 50% cytotoxicity (CC₅₀) was observed at a concentration of 1451.97 μg/ml. The selectivity index was calculated at 331 (Table 2).

To elucidate the mechanism of action of RMP, we examined whether it inhibited the binding of HIV-1 particles to MT-4 cells, as assessed by laser flow cytometry. It was found that RMP completely inhibited the binding of HIV-1 to MT-4 cells at a concentration of 100 μg/ml (Fig. 4).

The inhibitory activity of RMP against the formation of multinuclear giant cell (syncytium) in coculture of MOLT-4/HIV-1G12 and MOLT-4 cells was investigated. RMP inhibited the formation of syncytium with a 50% inhibitory concentration of 15.26 μg/ml (Fig. 5).

The infection of T-lymphocytes and macrophages by HIV is mediated by the binding of the HIV envelope
glycoprotein gp120 to a cell surface receptor CD4 molecule, which is an integral membrane glycoprotein of CD4+ cells.\(^{20,21}\) RMP completely blocked virus binding to the cells (Fig. 4) and the formation of syncytium upon cocultivation of MOLT-4/HIV-1\(_{\text{HIB}}\) cells and MOLT-4 cells, suggesting that RMP inhibits the binding of gp120 to CD4 molecules.

This study indicates that polysaccharide from \textit{Rhizophora mcronata} shows potent anti-HIV activity \textit{in vitro}, possibly due to interference with the adsorption of virus particle to CD4+ cells. The plant has been used in folklore medicine in India.\(^6\) We have previously reported that the extract of bark of \textit{R. mcronata} has \textit{in vitro} antiviral activity against Newcastle disease virus,\(^{22}\) vaccinia virus,\(^{23}\) encephalomyocarditis virus (EMCV),\(^{24}\) and Semliki forest virus (SFV)\(^{20}\) and the inhibitory activity against the binding of hepatitis B virus surface anti-

gen to its antibody.\(^7\) We reported that the extract protected Swiss albino mice from lethal infection by EMCV\(^{20}\) and SFV.\(^{25}\) Thus, the polysaccharide from \textit{Rhizophora mcronata} seems to be a new class of antiviral agent and a good candidate for therapeutic use against HIV and other viral infections. Further evaluations are underway to assess the efficacy and clinical usefulness of RMP.

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