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Antiviral properties of a mangrove plant, *Rhizophora apiculata* Blume, against human immunodeficiency virus

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Abstract

A polysaccharide extracted from the leaf of *Rhizophora apiculata* (RAP) was assessed in cell culture systems, for its activity against human and simian immunodeficiency viruses. RAP inhibited HIV-1 or HIV-2 or SIV strains in various cell cultures and assay systems. It blocked the expression of HIV-1 antigen in MT-4 cells and abolished the production of HIV-1 p24 antigen in peripheral blood mononuclear cells (PBMC); the 50% effective concentration (EC_{50}) of RAP in HIV-1 infected MT-4 cells and in PBMC was 10.7 and 25.9 μg/ml, respectively. RAP (100 μg/ml) completely blocked the binding of HIV-1 virions to MT-4 cells. RAP also reduced the production of viral mRNA when added before virus adsorption. RAP inhibited syncytium formation in cocultures of MOLT-4 cells and MOLT-4/HIV-1_{IIIB} cells. RAP did not prolong activated partial thromboplastin time (APTT) up to 500 μg/ml. These properties may be advantageous should RAP be considered for further development.

Keywords: *Rhizophora apiculata*; Rhizophoraceae; Polysaccharide; Human immunodeficiency virus; Anti-HIV activity; Indirect immunofluorescence assay

1. Introduction

Acquired immunodeficiency syndrome (AIDS), due to infection with the human immunodeficiency virus (HIV), has become a world-wide epidemic. Without effective preventive measures, a continuing AIDS pandemic is anticipated. Exist-

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ing therapies are targeted at the virus-associated reverse transcriptase and protease activities as a strategy to inhibit viral replication. The emergence of drug-resistant virus strains (Vandamme et al., 1994; Condra et al., 1995) and the occurrence of side effects (Richman et al., 1987; Hayakawa et al., 1991; Lang et al., 1993) are major disadvantages of the current therapies for the treatment of AIDS. Nevertheless, it is important to consider not only treatment of patients already infected with the virus, but also chemoprophylaxis and protection from new infections. Heterosexual transmission is responsible for the large majority of infections. It is essential to develop effective prevention strategies to combat further spread of the epidemic via this route. Earlier studies demonstrated that sulfated polysaccharides could be considered for a vaginal anti-HIV formulation (Pearce-Pratt and Phillips, 1993; Stafford et al., 1997).

Mangrove plants have been used in folklore medicine for several diseases in India (Kirtikar and Basu, 1935). We report here that an alkaline extract from leaf of *Rhizophora apiculata* (Rhizophoraceae) inhibits the HIV replication and HIV-induced cytopathic effects. The extract showed broad spectrum anti-HIV activity against T-cell tropic and macrophage tropic strains in different cell cultures. The substance responsible for the anti-HIV activity of the plant extract is an acid polysaccharide mainly composed of galactose, galactosamine and uronic acid.

2. Materials and methods

2.1. Reagents and chemicals

The following reagents were obtained from the indicated companies: dextran sulfate (8 kDa) (Kowa, Tokyo, Japan); 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, Osaka, Japan); DEAE-dextran (Pharmacia, Uppsala, Sweden); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Cappel Organon Teknika, West Chester, PA).

2.1.1. Purification of the polysaccharide from leaf of Rhizophora apiculata

Leaves of *R. apiculata* Blume were 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. Powdered leaf material was first extracted with hot water at 85°C for 3 h, and further extracted twice with 1% sodium carbonate at 45°C for 3 h. The alkaline extract was filtered through two layers of gauze, and designated as 'crude extract'.

The crude extract was fractionated in the presence of sodium acetate. The extract (1–5 mg/ml) was brought into 0.1 M sodium acetate, and then 1/4 volume of ethanol (25% ethanol) was added, and the mixture was allowed to stand for 1 h at 4°C and centrifuged at $150 \times g$ for 15 min. The precipitated fraction was designated as 0–25P. An equal volume of ethanol (50% ethanol) was added to the supernatant, and the mixture was processed as described above and designated as 25–50P. The supernatant obtained was further fractionated with the addition of a 3-fold volume of ethanol (75% ethanol) 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.

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

Column-purified sample was dissolved in distilled water. It could be filtered through millipore filters (Nihon Millipore Ltd., Yonezawa, Japan).

2.1.2. Analytical methods

The contents of neutral sugars and uronic acids were estimated by the phenol-sulfuric acid method (Dubois et al., 1956) and the carbazole method (Bitter and Muir, 1962), respectively. The contents of protein and sulfate were estimated as described by Bradford (1976) and Dodgson (1961), respectively.

The composition of sugars was analyzed by HPLC using Dionex chromatography model CRB (Dionex, Sunnyvale, CA) with Carbopac PA10 column (4×250 mm) after acid hydrolysis with 2 *N* HCl.

2.2. 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 3×10^5 cells/ml in RPMI 1640 medium supplemented with 10% (*V*/*V*) heat inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. MAGI-CCR5 cells (Chackerian et al., 1997) were cultured in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 300 μg/ml L-glutamine, 200 μg/ml G418, 100 μg/ml hygromycin B and 1 μg/ml puromycin. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and isolated by Ficoll–Hypaque gradient centrifugation and stimulated in RPMI 1640 medium containing 20% FCS, antibiotics and phytohemagglutinin (PHA, 5 μg/ml) for 3 days and cultured in RPMI 1640 medium containing 20% FCS, antibiotics and 5 ng/ml recombinant interleukin 2 (IL-2). A strain of HIV-1_{IIIB} was prepared from the culture supernatant of MOLT-4/HIV-1_{IIIB} cells, that were persistently infected with HIV-1_{IIIB}. Nevirapine-resistant mutant HIV-1 strain HIV-$1_{HE\text{-}NEV}$ (Baba et al., 1994) and MKC-442-resistant strain HIV-$1_{IIIB\text{-}R}$ (Seki et al., 1995), HIV-2_{ROD} and SIV_{MAC} were maintained in MT-4 cells. Macrophage tropic (M-tropic) virus strain HIV-$1_{JR\text{-}FL}$ was cultured in human peripheral blood macrophages.

2.2.1. Antiviral assay

Anti-HIV and anti-SIV activities of the test substance were based on the inhibition of virus-induced cytopathogenicity in MT-4 cells, as determined by the MTT method (Pauwels et al., 1988; Premanathan et al., 1996). The anti-HIV-1 efficacy was also assessed from the inhibitory effect on virus-specific antigen expression, as determined by an indirect immunofluorescence method (Premanathan et al., 1997).

The activity against M-tropic strain HIV-$1_{JR\text{-}FL}$ was assessed in the MAGI assay, as described elsewhere (Kimpton and Emerman, 1992; Premanathan et al., 1998). The assay procedure for measuring the anti-HIV activity of the test substance in PBMC was based on the quantitative detection of p24, which was determined with a sandwich enzyme-linked immunosorbent assay kit (Abbott GmgH Diagnostika, Wiesbaden-Delkenheim, Germany) after 16 days of infection (Premanathan et al., 1998). Cytotoxicity of RAP in MT-4, MAGI-CCR5 and PBMC was evaluated by the viability of mock-infected cells, as determined by MTT, trypan blue stain and [^{3}H]thymidine incorporation, respectively.

2.2.2. Syncytium formation assay

MOLT-4 cells (5×10^5) were cultured with the equal number of MOLT-4/HIV-1_{IIIB} cells in microtiter plate wells containing various concentrations of the test substance. After 20 h of cocultivation, the number of viable cells was determined by the trypan blue dye exclusion method, and the fusion index (FI) was calculated as described earlier (Tochikura et al., 1988).

2.2.3. Assay for virion binding to MT-4 cells

Virion binding to the cell membrane was determined by an indirect immunofluorescence assay using polyclonal antibody derived from a patient with AIDS-related complex, FITC-rabbit anti-human IgG, and flow cytometry (Schols et al., 1989; Premanathan et al., 1997).

2.2.4. Monoclonal antibody binding assay

MT-4 cells or PBMC were incubated with the test substance (100 μg/ml) for 30 min at 4°C and then either stained with FITC conjugated anti-

CD4 monoclonal antibody (mAb) (MT310) (Dako, Denmark) or incubated for 30 min at 4°C with either anti-human CXCR4 mAb (12G5) (R&D Systems, Minneapolis, MI) or anti-human CCR5 mAb (2D7) (NIH AIDS Research and Reference Reagent Programs) and stained with-FITC-conjugated anti-mouse IgG (Cappel Organon Teknika, West Chester, PA), and analyzed cytofluorometrically.

2.2.5. Competitive reverse transcriptase-polymerase chain reaction amplification

MT-4 cells were treated with virus (m.o.i. = 1) and incubated for 90 min at 37°C for virus adsorption with or without test substance. They were then washed three times with medium and further incubated with medium containing the test substance. After 12 h, total mRNA was extracted from 10^6 cells by a standard protocol using DYNAL Dynabeads mRNA direct kit (Dynal A.S, Oslo, Norway). Competitive RT-PCR was performed for mRNA amplification by the Takara competitive RNA transcription kit (Takara Shuzo, Tokyo, Japan), using self designed forward upstream primer 5′-CTACCACACACAAGGCT-3′ and reverse downstream primer 5′-GGTCTGAGGGATCTCTA-3′ of the LTR region of the HIV genome.

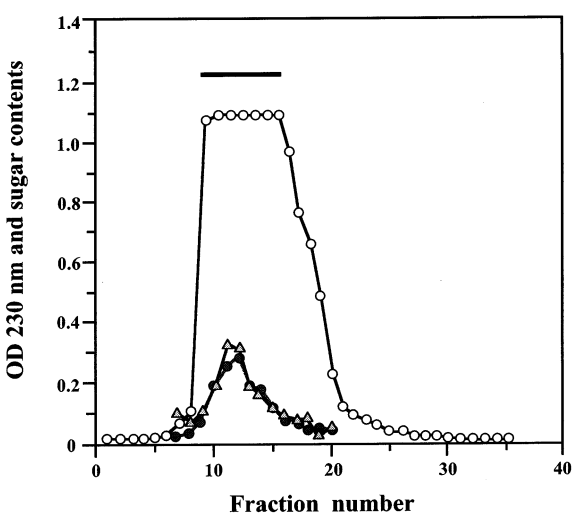

Fig. 1. Cellulofine GC-700m column chromatography of the alkaline extract from *Rhizophora apiculata*. ○, OD_{230} nm; ●, neutral sugars; △, uronic acids.

2.3. Activated partial thromboplastin time (APTT)

The APTT of plasma from a healthy volunteer was determined in the presence of the test substance by using an automated machine (CA-5000, Toa Medical Electronics). The test substance (40 μl) was mixed with 360 μl of normal human plasma and then the APTT test was performed.

3. Results

3.1. Purification of polysaccharide from Rhizophora apiculata (RAP)

The plant polysaccharide was precipitated in ethanol, in the presence of 0.1 M sodium acetate. The fraction 25–75P showed higher activity than the 0–25P and 75S fractions (data not shown). This active fraction was further analyzed for its chromatographic profile on a column of Cellulofine GC-700m. There was a peak at fraction number 9–15 (Fig. 1). These fractions were combined, and designated as RAP and used for further studies.

3.2. Sugar composition of RAP

The column-purified polysaccharide from *R. apiculata* (RAP) was analyzed for its composition. It was found that RAP consisted of 41, 17 and 27% of neutral sugars, uronic acid A and B, respectively (Table 1). The neutral sugars alone were further analyzed for their composition by using HPLC and found to be composed mainly of galactose (47 mol%) and other components such as galactosamine, glucose and arabinose (Table 1).

3.3. In vitro anti-HIV activity of RAP

The anti-HIV-1 activity of RAP was assessed by protection against HIV-1-induced cytopathogenicity and inhibition of virus-specific antigen

Table 1
Components of the alkaline extracts from *Rhizophora apiculata* leafs

Component[a] *(mg/100 mg)*	
Neutral sugars	41.43 ± 10.98
Uronic acid (A)	17.24 ± 2.48
Uronic acid (B)	27.54 ± 4.09
Sugar component[b] *(mol%)*	
Galactose	46.76
Galactosamine	19.00
Glucose	17.14
Arabinose	17.11

[a] Uronic acids were estimated by the carbazole method, using glucuronic acid (A) and galacturonic acid (B) as the standards.

[b] Sugar components of the purified polysaccharide estimated by HPLC.

expression in MT-4 cells in vitro (Fig. 2). RAP showed concentration-dependent inhibition of HIV-1 with a 50% effective concentration (EC_{50}) of 6.5 μg/ml. Fifty percent cytotoxicity (CC_{50}) was observed at a concentration of 1545 μg/ml. The selectivity index (CC_{50}/EC_{50}) was calculated as 237. In the same culture condition, dextran sulfate inhibited HIV-1 replication with an EC_{50} value of 5.3 μg/ml. Antiviral activity of RAP was observed with several strains of HIV-1, HIV-2 and SIV in MT-4, PBMC and MAGI-CCR5 cells. Irrespective of the criteria used to assess antiviral activity, i.e. inhibition of viral cytopathogenicity by MTT assay, antigen expression, p24 production, or the MAGI assay, RAP inhibited HIV-1 or HIV-2 or SIV replication within the EC_{50} concentration range of 6.5 to 40.6 μg/ml (Table 2).

To elucidate the range of molecular weight responsible for anti-HIV activity of RAP, molecular weight filtration study was conducted. Anti-HIV activity was retained with the $>30\,000$ MW fraction (data not shown).

3.4. Inhibition of syncytium formation

The inhibitory activity of RAP against the formation of multinuclear giant cells (syncytia) in cocultures of MOLT-4 and MOLT-4/HIV-1_{IIIB} cells was investigated. RAP inhibited syncytium formation with a 50% inhibitory concentration of 53.3 μg/ml (Fig. 3). Under the same culture conditions, dextran sulfate inhibited syncytium formation with an EC_{50} of 13.5 μg/ml.

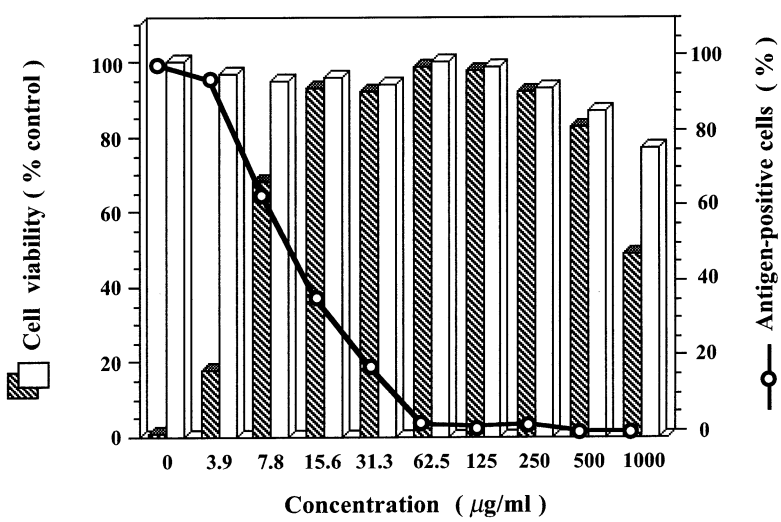

Fig. 2. Anti-HIV-1 activity and viral antigen inhibition in MT-4 cells by polysaccharide from *Rhizophora apiculata* (RAP). The viability of HIV-infected MT-4 cells (hatched columns) and mock-infected MT-4 cells (open columns) was measured by the MTT method. The number of viable cells is expressed as a percentage of mock-infected drug-free control cells. HIV-1 antigen-positive cells were detected by indirect immunofluorescence staining, using a polyclonal antibody as a probe. The number of viral antigen-positive cells is expressed as percentage of the HIV-infected drug-free control cells.

Table 2
Anti-HIV activity of the column chromatography-purified alkaline extract of *Rhizophora apiculata*[a]

Virus strain	Cells	Assay	Day of analysis	EC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
HIV-1						
IIIB	MT-4	MTT	5	6.5 ± 2.0	1545.7 ± 272.3	237.8
IIIB	MT-4	Ag expression	3	10.7 ± 3.9		144.5
IIIB	PBMC	p24	16	25.9 ± 4.2	1136.9 ± 180.3	44.0
IIIB-R[b]	MT-4	MTT	5	28.6 ± 2.6		54.0
HE-NEV[c]	MT-4	MTT	5	40.6 ± 6.4		38.1
JR-FL[d]	MAGI-CCR5	MAGI	3	16.6 ± 2.6	1427.1 ± 336.1	86.0
HIV-2						
ROD	MT-4	MTT	5	10.2 ± 4.6		151.5
SIV						
MAC	MT-4	MTT	5	24.3 ± 3.7		63.6

[a] The EC_{50} is calculated on the basis of the inhibition of HIV-induced cytopathogenicity, or HIV antigen expression in MT-4 cells, or reduction of blue cells in the MAGI assay, or the reduction of p24 antigen in the culture supernatant of PBMC. The CC_{50} is calculated on the basis of the reduction of the viability of mock-infected cells. Data represent the mean values with standard deviations for at least three separate experiments. SI, selectivity index (CC_{50}/EC_{50}); Ag, antigen.
[b] MKC-442-resistant HIV-1.
[c] Nevirapine-resistant HIV-1.
[d] Macrophage-tropic HIV-1.

3.5. Inhibition of virus binding to MT-4 cells

To elucidate the mechanism of action of RAP, we examined whether it inhibited the binding of HIV-1 particles to MT-4 cells, as assessed by flowcytometry. RAP completely inhibited the binding of HIV-1 to MT-4 cells at a concentration of 100 μg/ml (Fig. 4). However, at the same concentration RAP did not inhibit the binding of mAbs to CD4, CXCR4 in MT-4 cells or mAbs to CCR5 in PBMC.

3.6. Competitive RT-PCR analysis

To determine whether RAP inhibited the expression of mRNA of HIV-1, we determined the amount of viral mRNA expressed in the cells employing competitive RT-PCR using self designed primers for the LTR region. HIV-1 mRNA reached 10^7 copies in the control virus-infected cells, whereas treatment with RAP (100 μg/ml) at the time of virus adsorption reduced the number of copies of viral mRNA to less than 10^2 copies (Fig. 5). However, there was no reduction in mRNA copies when RAP was added after virus adsorption (data not shown).

3.7. Anticoagulant activity of RAP

The effect of RAP on APTT of human plasma was analyzed using an automated machine. RAP did not prolong APTT at concentrations up to 500 μg/ml. However, heparin and dextran sulfate

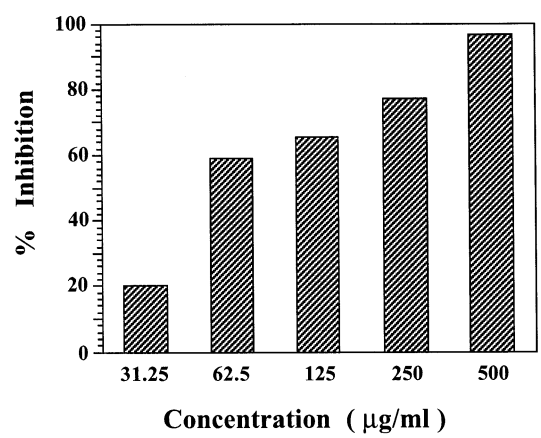

Fig. 3. Inhibitory effect of RAP on syncytium formation in a coculture of MOLT-4 and MOLT-4/HIV-1_{IIIB} cells. The number of viable cells was determined after 20 h of coculture in the presence of various concentrations of RAP, and percentage of fusion inhibition was calculated.

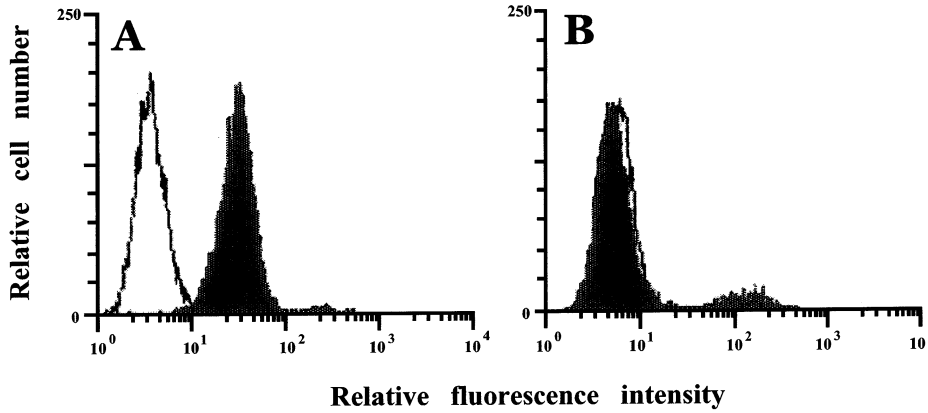

Fig. 4. Effect of RAP on HIV-1 binding to MT-4 cells. The thick-line histograms represent cellular fluorescence resulting from non-specific binding of anti-HIV-1 antibody to MT-4 cells (which had not been exposed to HIV-1). The thin-line histograms with shading represent the cellular fluorescence resulting from specific binding of anti-HIV-1 antibody to virus-exposed MT-4 cells in the absence of the test compound (A), or in the presence of RAP (100 μg/ml) (B).

markedly prolonged APTT (Table 3). These findings suggest that RAP would have only weak anticoagulant activity.

4. Discussion

The present study indicates that a polysaccharide extracted from *R. apiculata* (RAP) has anti-HIV activity in vitro, apparently due to interference with the adsorption of virus particles to CD4-positive cells.

The anti-HIV activity from *R. apiculata* was mainly recovered in fraction 25–75P by ethanol precipitation, and the extract contained a large amount of neutral sugars and uronic acids. No protein or sulfate could be detected. The acid polysaccharide from *R. apiculata* had a molecular weight of more than 30 000 and was mainly composed of galactose, galactosamine and uronic acid. Its antiviral activity may be attributed to its carboxylated (polyanionic) character.

The infection of T lymphocytes and macrophages by HIV is mediated by the binding of the HIV envelope glycoprotein gp120 to cell surface receptor CD4 molecule which is an integral membrane glycoprotein of CD4-positive cells (Dalgleish et al., 1984; Klatzmann et al., 1984). RAP completely blocked virus binding to the cells at a concentration of 100 μg/ml (Fig. 4). At the same concentration it did not influence the binding of mAbs against cellular membrane surface proteins. RAP also abolished syncytium formation upon cocultivation of MOLT-4 cells and MOLT-4/HIV-1_{IIIB} cells, suggesting that it would interfere with gp120 and prevent the formation of gp120/CD4 complex. This was also confirmed by competitive RT-PCR analysis: viral mRNA was markedly reduced by RAP when added before virus adsorption (Fig. 5), but there was no reduction in mRNA copies when RAP was added after virus adsorption (data not shown). All the data obtained with RAP clearly point to the attachment of the virus to the cell membrane as the target of its antiviral action. RAP may be assumed to block this attachment through formation of a shield between the viral envelope gp120 glycoprotein and the cellular membrane CD4 receptor.

It is important to consider not only treatment of patients already infected with the virus, but also chemoprophylaxis and protection from new infection. Heterosexual transmission is responsible for the large majority of infections. It is essential to develop effective prevention strategies to combat further spread of the epidemic through this route. Because of poor oral absorption (Lorentsen et al., 1989; Hartman et al., 1990) and high anti-

coagulant activity (Bagasra and Lischner, 1988; Baba et al., 1990), sulfated polysaccharides failed to reach clinical trials as an oral drug. However, earlier studies demonstrated that sulfated polysaccharides can be used as a vaginal anti-HIV formu-lation without disturbing the function of the vaginal epithelial cells and normal bacterial flora (Pearce-Pratt and Phillips 1993; Stafford et al., 1997). RAP inhibits transmission of free infectious virions to the cells as well as cell-to-cell transmission, and could be considered for further development as a vaginal anti-HIV formulation. The duration of contact between virus and the drug is short upon topical application and this may prevent the emergence of drug-resistant HIV.

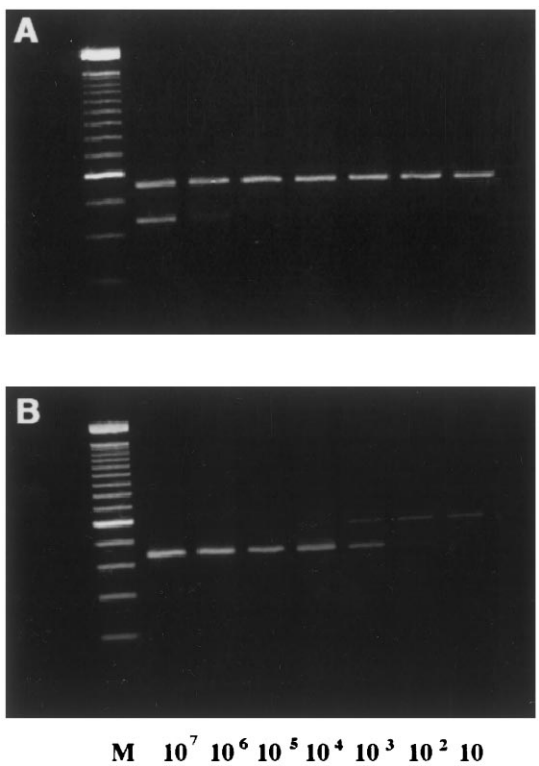

Fig. 5. Competitive RT-PCR. MT-4 cells were exposed to HIV-1 with or without RAP (100 μg/ml) for 90 min and washed thoroughly to remove unadsorbed virus. mRNA was isolated from 10^6 cells at 12 h of incubation with or without RAP. Competitive RT-PCR was performed as described in Section 2. (A) virus control, (B) treated with RAP. Numbers at the bottom denote the number of competitive RNA copies in each lane.

Table 3
Effect of the polysaccharide from *Rhizophora apiculata* on activated partial thromboplastin time (APTT)

Compound	Concentration (μg/ml)	APTT[a] (s)
R. apiculata	1000	139.8
	500	53
	250	36.4
	125	32.5
Dextran sulfate	25	> 1260
	12.5	195.6
	6.125	101.7
Heparin (U/ml)	10	>1260
	1	309.6
	0.1	36.4
Control		33.8

[a] Activated partial thromboplastin time of normal human plasma in the presence of test substance.

RAP did not show cytotoxicity at a concentration less than 1000 μg/ml, and showed anti-HIV activity at a relatively low concentration against M- and T-tropic virus strains (Table 2). RAP did not prolong the APTT up to 500 μg/ml. RAP contains neither protein nor sulfate. Thus, the polysaccharide from *R. apiculata* seems to represent a different class of anti-HIV agents. Further studies on pharmacokinetics, spermicidal activity, tolerance by the genital mucosa, and effects on the normal vaginal flora are under progress.

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MAGI-CCR5 from Dr Julie Overbaugh; CCR5 monoclonal antibody (2D7) from LeukoSite, Inc.

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