Inhibition of HIV-1 Replication by an Aqueous Extract of Spirulina platensis (Arthrospira platensis)

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Summary: An aqueous extract of the blue-green filamentous algae Arthrospira platensis (previously called Spirulina platensis) inhibited HIV-1 replication in human T-cell lines, peripheral blood mononuclear cells (PBMC), and Langerhans cells (LC). Extract concentrations ranging between 0.3 and 1.2 μg/ml reduced viral production by approximately 50% (50% effective concentration [EC50]) in PBMCs. The 50% inhibitory concentration (IC50) of extract for PBMC growth ranged between 0.8 and 3.1 mg/ml. Depending on the cell type used, therapeutic indices ranged between 200 and 6000. The extract inactivated HIV-1 infectivity directly when preincubated with virus before addition to human T-cell lines. Fractionation of the extract revealed antiviral activity in the polysaccharide fraction and also in a fraction depleted of polysaccharides and tannins. We conclude that aqueous A platensis extracts contain antiretroviral activity that may be of potential clinical interest. Key Words: Arthrospira platensis extract—HIV-1 replication inhibition—Primary cells.

HIV-1, the causative agent of AIDS, is spreading rapidly. Although newer drug combination regimens show promise, problems remain. 3'-Azido-3'-deoxythymidine (zidovudine, AZT) may become ineffective because of the emergence of resistant strains (1,2). More recently approved drugs, such as protease inhibitors or novel inhibitors of reverse transcriptase, and combination drug therapies are expensive. As the AIDS epidemic continues to escalate, the development of new drugs for treatment of HIV-1-infected individuals remains a challenge. One possible approach could be to screen natural products for anti-HIV-1 activity. Although the search for anti-HIV-1 compounds from natural sources has been extensive, success has been rare. Positive results have been reported from extracts of blue-green algae (e.g., Lyngbya lagerheimii, Phormidium tenue [3]), the brown seaweed Fucus vesiculosus [4]). However, mass production of the required substances is limited by the availability and maintenance of these organisms on a large scale. In this report, we evaluated the anti-HIV-1 activity of a hot-water extract from the commercially produced algae, Spirulina platensis, now renamed Arthrospira platensis.

A platensis, a blue-green filamentous alga that grows in alkaline lakes, has a high protein content and has been used as animal feed. Administration of A platensis in a human diet at 10 to 12 g/meal has been reported (5). Toxicity studies have been performed in mice, rats, and hamsters (6–8), and inhibition of herpes simplex virus type 1 (HSV-1) with aqueous extracts of A platensis has been demonstrated (6). The various uses of A platensis have been reviewed by Belay et al. (9,10).

The objective of this study was to evaluate the effect of aqueous extracts of A platensis on the replication of HIV-1 in vitro. We observed that hot-water extracts of this organism inhibit HIV-1 replication and syncytium formation at concentrations found to be nontoxic to human cells.
MATERIALS AND METHODS

HIV-1 Stocks

The HIV-1 strains (HIV-IIB, HIV18, and 11G, a patient isolate) were propagated either in the Jurkat T-cell line or human peripheral blood mononuclear cells (PBMC).

Cytotoxicity Assays

Human T-cell lines (Jurkat, CEM-SS) and human PBMC (1 x 10⁴ to 4 x 10⁵ cells) were seeded into microtiter plates and cultured for 1 to 7 days in the presence of various concentrations of algal extract. The medium was removed gently, the number of viable cells was determined by trypan blue exclusion or XTT assay (discussed later) (11), and the 50% inhibitory concentration (IC₅₀) for the extract was calculated.

Measurement of Antiviral Activity

p24 Gag Antigen Determination in Culture Supernatants

Jurkat cells or PBMC were seeded in 24-well plates with various dilutions of extract. Four hours later, the cells were washed, pulsed for 1 to 24 hours with HIV-1 at a multiplicity of infection of 0.1 in the continued presence of extract. The cells were washed 4 times and grown in medium containing the appropriate dilutions of the extract, which was replaced every 3 days. Supernatants were collected and frozen at -70°C. p24 Antigen levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DuPont, Billerica, MA, U.S.A.) according to the manufacturer’s instructions.

Anti-HIV-1 Screening Using the XTT Assay

Antiviral and cytotoxic effects of the extract were evaluated simultaneously with the XTT assay (11). Quadruplicate wells of U-shaped 96-well microtiter plates containing 1 x 10⁴ CEM-SS cells (100 µl) were incubated with 100-µl dilutions of extract for 4 hours, followed by addition of 50 µl of virus. After 7 days of culture, 50 µl of a mixture of 1 mg/ml of XTT and 0.02 mM ß-methylphenazonium methosulfate were added, incubated for 4 hours to allow XTT-formazan production, and the optical density (OD) was determined at a test wavelength of 450 nm and a reference wavelength of 650 nm. Only live, uninfected cells or cells protected from HIV-1 lysis by the extract produce an orange XTT formazan product, and thus a high OD value. The 50% effective concentration (EC₅₀) is defined as the concentration of extract that increases XTT formazan production in infected cultures to 50% of that of untreated, uninfected control cells, after subtraction of background absorption of infected, untreated samples. The IC₅₀ of the extract is defined as the mean value of the concentration of extract that inhibits the XTT formazan production in uninfected control cells to 50% of the mean value obtained for untreated, uninfected control cells; because higher extract concentrations showed absorption at 450 nm, this background OD of extract in medium only was subtracted. The therapeutic index is defined as the ratio of the IC₅₀/EC₅₀. To test aqueous extracts after further fractionation that removed polysaccharides and tannins, the cells were exposed to the test material for 3 hours, followed by virus infection.

Inhibition of Syncytium Formation

This test was performed as described by Nara et al. (12). Flat-bottom, 96-well plates were coated with 50-µg/ml, 50-µl/well poly-L-lysine, incubated at room temperature for 60 minutes, and washed twice with phosphate-buffered saline. CEM-SS cells (1 x 10⁶ cells/well in growth medium containing 25 µg/ml of diethylaminoethyl-dextran (DEAE-dextran), were allowed to adhere for 30 minutes at 37°C. 5% CO₂. DEAE-dextran was aspirated, and 40 µl of virus supernatant obtained from the XTT culture on day 7 were added to the cells, incubated for 60 minutes, and replaced with 100 µl of growth medium. After 5 days, syncytia were counted manually. Five wells containing uninfected CEM-SS cells were included as negative control.

Inhibition of HIV-1 Transfer From Langerhans Cells to Uninfected T Cells

Human skin Langerhans cells (LC) and T cells were isolated as described (13). The ability of LC to transfer HIV-1 to uninfected target T cells was examined in the presence of algal extract. LC were preincubated with 100 µl of extract for 2 hours at 37°C, pulsed with HIV-1 for 16 hours at 37°C, washed 4 times and cocultured with uninfected allogeneic T cells. Every 3 to 4 days, culture supernatants were collected for p24 antigen assay.

Extract Preparation

Five grams of dehydrated A. platensis powder (Earthrise Farms, Calipatria, CA, U.S.A.) were resuspended in distilled water (10 ml/g), mixed, extracted with boiling water for 60 minutes, and centrifuged twice at 8000 rpm for 10 minutes. The supernatant was lyophilized to yield 0.2-0.44 g of a blue powder, which was resuspended in growth medium (RPMI-1640 supplemented with 10% fetal bovine serum [FBS], penicillin, streptomycin, and l-glutamine). To remove anionic polysaccharides, the aqueous phase was processed as published (14) (Fig. 1). Briefly, lyophilized aqueous extract was dissolved in dH₂O at 50 mg/ml, precipitated with an equal volume of absolute ethanol (EtOH), and stored at -20°C overnight, followed by centrifugation at 1000 rpm. The precipitate (i.e., the polysaccharide fraction) was lyophilized. EtOH was removed from the supernatant by rotary evaporation, 25 mg of the lyophilized residue was dissolved in 2 ml of dH₂O, and fractionated further by using a polyamide column preswollen in water overnight.

To remove tannins (polyphenols) that are known to be irreversibly retained on polyamide resins (15), Mackerey Nagel polyamide SC6 was preswollen with dH₂O overnight (1 g for every 5 mg of lyophilized supernatant) and packed into a 35-ml syringe fitted with a glass wool plug in the tip. The material (25 mg in 2 ml of dH₂O) was applied to the column and eluted in four fractions: 10 ml of dH₂O, 10 ml dH₂O/methanol (1:1), 20 ml methanol, and 20 ml methanol. Each fraction was evaporated using the rotary vaporizer and dissolved in 1.2 ml of RPMI-1640 medium containing 10% FBS and penicillin/streptomycin. Fractions A and B were colorless, fraction C was yellow-green, and fraction D was light yellow-green to pink.

RESULTS

First, we evaluated the effects of increasing concentrations of the algal extract on HIV-1 replication in Jurkat cell cultures by microscopic examination for syncy-
Next, the aqueous algal extract was tested for inhibition of HIV_{RF} replication in human CEM-SS cells and PBMC. The EC_{50} ranged between 0.3 and 1.2 µg/ml, a value that approximated that seen in Jurkat cells (Table 1).

### Evaluation of Anti–HIV-1 Activity by XTT Assay

Inhibition of cell growth and viral replication was evaluated simultaneously by a second, independent method, the XTT assay (Fig. 2). The EC_{50} in this system was 24 µg/ml. The increase in XTT formazan production in infected cultures depended on extract concentration. At lower concentrations, no inhibition of viral production was observed, and as a result, the target cells died, leading to low OD levels. However, at concentrations that are toxic to cells as measured by trypan blue exclusion, the OD reading was higher than expected. For instance, at an extract concentration of 6.25 mg/ml, 62.5% of the cells were dead by trypan blue exclusion, whereas the OD reading at that concentration corresponded to a viability of 94.5%. We ascribe the inappropriately high OD values to the adsorption of the extract itself at the test wavelength (data not shown); consequently, the baseline OD values obtained for extract in medium only were subtracted (see Methods). Furthermore, supernatants were obtained from these cultures on day 7, added to fresh CEM-SS cells, and analyzed for inhibition of syncytium formation. Results were in agreement with the XTT assay (data not shown).

### TABLE 1. Summary of the anti–HIV-1 activity of Arthrospira platensis aqueous extracts in various cell types

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Virus strain</th>
<th>Cells</th>
<th>Assay</th>
<th>EC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IIIB</td>
<td>Jurkat</td>
<td>p24 (day 5)</td>
<td>0.01–0.10</td>
</tr>
<tr>
<td>2</td>
<td>RF</td>
<td>CEM-SS</td>
<td>Syncytium formation (day 5) after preincubation of virus with extract only</td>
<td>3–12</td>
</tr>
<tr>
<td>3</td>
<td>RF</td>
<td>PBMC</td>
<td>p24 (day 7)</td>
<td>0.3–1.2</td>
</tr>
</tbody>
</table>

Two different extracts were used for the various experimental series (experiment 1; experiments 2 and 3). The data are representative of one of two similar experiments for each series. Experiment 1: With or without pretreatment with various concentrations of extract for 3 hours, Jurkat cells were pulsed with HIV–IIIB for 24 hours in the presence of algal extract at the appropriate concentrations. After washing, the cultures were maintained for 5 days in the presence of the extract, at which time p24 antigen levels were determined in the supernatants. Experiment 2: Pretreatment of HIV_{RF} with extract. HIV_{RF} was incubated with various concentrations of extract for 60 minutes at room temperature and added to CEM-SS cells for 24 hours. The cells were washed 4 times, and the cultures were maintained for 7 days in the absence of extract. Supernatants were collected and syncytia were counted on day 5. Experiment 3: PBMC were pretreated with various concentrations of extract for 4 hours, pulsed with HIV_{RF} for 24 hours, and washed. Cultures were maintained in the presence of extract. p24 levels measured on day 7 were used to estimate the EC_{50}. EC_{50}, 50% effective concentration; PBMC, peripheral blood mononuclear cells.
Clearly, the one-time exposure of virus to extract resulted in diminished virus infectivity. Syncytium formation was blocked completely when virus had been treated once with >48 μg/ml of extract (data not shown).

Furthermore, to gain some information regarding the mechanism of HIV-1 inhibition by the aqueous extract, we examined its effect on late steps in the viral life cycle. To do this, we used the chronically infected cell lines ACH2 and U1 which release infectious virus when cultured with tumor necrosis factor-α (TNF-α). On stimulation of these cells with TNF-α, virus production was not affected by the presence of the algal extract, indicating that the latter has no significant effect on postintegration steps in the virus life cycle (data not shown).

**Fractionation of the Aqueous Extract to Remove Polysaccharides and Tannins**

To eliminate tannins and polyphenols (known to be prevalent especially in higher plant extracts and considered to be nonspecific inhibitors), and to identify the fractions that contain the anti–HIV-1 activity, a two-step procedure was introduced. First, the anionic polysaccharide portion was removed by EtOH precipitation. Second, tannins and polyphenols were removed by passing over polyamide resins. The polyamide column eluate was collected in four different fractions, each of which was tested for anti–HIV-1 activity.

The EtOH precipitate fraction (mainly polysaccharides; Fig. 1) contained potent anti–HIV-1 activity with an

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**Inhibition of a Primary HIV-1 Isolate in Langerhans Cells**

Because HIV-1 isolates vary in their cellular tropism, we evaluated the effect of the aqueous extract on the primary patient isolate, 11G, using primary human LC as targets for HIV-1 infection. Mucosal LC are considered to be the primary target cells during heterosexual transmission of HIV-1 and may represent virus reservoirs in chronically infected individuals (16). Here, LC pretreated with the aqueous extract were pulsed with HIV-1 11G in the presence of extract and washed extensively. Then, the virus–exposed LC were cocultured with mitogen-activated, uninfected human T cells in the absence of extract. As shown in Figure 3, the brief treatment of the LC/HIV-1 mixture with extract prevented virus transmission to susceptible T cells; cytotoxicity was negligible at this extract concentration.

**Direct Antiviral Effects**

To test whether the aqueous extract had direct antiviral effects, HIV<sub>Ref</sub> was preincubated with various concentrations of aqueous extract, followed by the addition of the mixture to CEM-SS cells (Table 1, experiment 2).
EC₅₀ of approximately 40 μg/ml; 100 and 200 μg/ml of the precipitate completely inhibited syncytium formation (data not shown). The EtOH supernatant portion of the extract also inhibited HIV-1 replication and syncytium formation (Table 2). This EtOH supernatant was fractionated further by using a polyamide column to trap tannins and polyphenols. The column efflux was collected in four different fractions that were tested for HIV-1 inhibitory activity. The water-methanol fraction (fraction B) inhibited HIV-1 replication by 50% to 70% in the XTT assay and reduced syncytium formation. Fractions C and D were entirely inactive. In some preparations, fraction A also had anti–HIV-1 activity (data not shown).

**DISCUSSION**

We have demonstrated that a hot-water extract of *A. platensis* inhibited HIV-1 replication and syncytium formation using two human T-cell lines, primary PBMC, and LC. T-cell–tropic viruses (HIV-1IB and HIVRF) and a primary patient isolate (11G) were inhibited. A polysaccharide-containing fraction as well as a tannin-free fraction were responsible for this anti–HIV-1 activity.

Although the antiviral mechanisms of this aqueous extract remain to be elucidated pending the complete purification of the active compound or compounds, inhibition of virus replication and syncytium formation may occur through binding of the polysaccharides to the CD4 receptor resulting in the disruption of CD4-gp120 interactions (17); direct effect on the virus reverse transcriptase similar to other blue-green algal extracts that inhibit the avian myeloblastoma virus (AMV) and HIV-1 (18); or a combination of both mechanisms.

<table>
<thead>
<tr>
<th>TABLE 2.</th>
<th>Inhibition of HIV₅₀ by ethanol (EtOH)-supernatant fraction of extract</th>
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<tbody>
<tr>
<td>Concentration (μg/ml)</td>
<td>Viral inhibition (%)</td>
</tr>
<tr>
<td>500</td>
<td>96</td>
</tr>
<tr>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>125</td>
<td>100</td>
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<tr>
<td>25</td>
<td>15.2</td>
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<tr>
<td>12.5</td>
<td>0.00</td>
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</table>

The dried aqueous extract of *Arthrosperia platensis* was reconstituted in water and ethanol (1:1) and placed at –20°C overnight. The extract solution was centrifuged, and the precipitate (polysaccharide portion) was removed. Absolute ethanol (EtOH) was removed from the supernatant by rotary evaporation and the remaining portion was lyophilized. The EtOH-extracted fraction of the aqueous extract was reconstituted in medium, filtered, and tested for inhibition of HIV₅₀ by the XTT assay. Additionally, the number of syncytia were graded (–, negative; + to ++++, an increasing intensity in syncytium formation). The data are representative of one of two similar experiments.

To identify the fraction or fractions that contain antiviral activity, we further fractionated the aqueous extract by dissolving it in EtOH. This procedure enabled us to precipitate the polysaccharide portion from the remainder of the EtOH-soluble components. The polysaccharide fraction of *A. platensis* inhibited HIV-1 replication and syncytium formation. In accord with our observation, previous studies have reported that sulfated polysaccharides isolated from blue-green algae and marine microalgae possess potent antiviral activity (19–21). Sulfated polysaccharides, such as dextran sulfate, also inhibit HIV-1 binding to CD4 receptors (17).

Because tannins and their derivatives are known to inhibit HIV-1 replication or HIV-1 reverse transcription (22,23), we removed them from the EtOH-soluble fraction by irreversible binding to a polyamide column (15). Analysis of the four different fractions obtained from the column eluate revealed the presence of anti–HIV-1 activity in fraction B. This suggested that different fractions of the original hot-water extract are responsible for the antiviral effect that has been observed in our study. Isolation of the active ingredient or ingredients in the EtOH-soluble fraction of the extract that remains after the removal of tannins should be pursued.

Recently, Boyd et al. (24) isolated a novel HIV–inactivating protein from cultures of the cyanobacterium *Nostoc ellipsosporum*, a blue-green algae. The 11-kd antiviral protein prevented in vitro replication and cytopathic effects of primate lentiviruses, including simian immunodeficiency virus and diverse laboratory strains of HIV-1 and HIV-2. This antiviral effect was believed to result from the interaction of the antiviral protein with the gp120 component of the virus envelope glycoprotein. Whether a similar protein or other novel compounds are involved in the antiviral effect of the *A. platensis* extract warrants further investigation.

**Acknowledgments:** We thank Yuwen Hu for technical assistance and Anne DiSorbo for the preparation of this manuscript. This work was supported by a grant from Earthrise Farms (S.A., R.M.R.). T.W.B. is a Scholar of the Pediatric AIDS Foundation.

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