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Isolation, purification and partial characterization of an active anti-HIV compound from the Chinese medicinal herb *Viola yedoensis*

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Summary

The dimethylsulfoxide extract of the Chinese medicinal herb *Viola yedoensis* demonstrates high inhibitory activity toward HIV-1 in vitro. The corresponding methanol extract also showed inhibitory activity but not as high as the dimethylsulfoxide extract. Anti-HIV activity in the extracts is accompanied by cytotoxicity, and we describe here the separation of the cytotoxic material from the active fraction. We also describe the procedure for isolation, purification, and separation of the active component from crude extracts of *V. yedoensis* as well as details of its activity against HIV-1. The UV-visible, infrared (IR) and proton nuclear magnetic resonance (NMR) data and certain other characteristics of the active compound are also presented. Initial chemical tests and the proton NMR and IR spectra indicate a high molecular weight sulphonated carbohydrate polymer, and chromatographic evidence suggests an MW between 10000 and 15000.

Chinese herb; Polysaccharide; Anti-HIV compound; Purification; Chromatography; Spectroscopy

Introduction

Several Chinese medicinal herbs have been reported to have inhibitory activities against the human immunodeficiency virus (HIV-1) in vitro (Chang and Yeung,

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1988). Here we report our purification and characterization study of one of them, *Viola yedoensis*, which us used by herbalists in China for the treatment of influenza, pharyngitis, acute gastritis and acute hepatitis (Chinese People's Liberation Army, 1969). The plant is also called *Zornia diphylla* (L.) Pers. and has several other names (Chinese People's Liberation Army, 1969).

Materials and Methods

A number of methods were investigated in order to isolate the active component from the crude extract of *V. yedoensis*. These include the use of preparative reversed-phase thin layer chromatography (with different percentages of methanol and water mixture as the mobile phase), C-18 reversed-phase high performance liquid chromatography (HPLC), normal phase thin layer chromatography, and Sephadex G-100 gel filtration gravity chromatography. All showed unsatisfactory results. Finally, an acceptable isolation and purification procedure was developed by using a variety of different protic and aprotic solvents and a gel filtration HPLC column.

Isolation and purification of crude extract of V. yedoensis

An aqueous extract was prepared from *V. yedoensis* by a procedure described previously (Chang and Yeung, 1988). Briefly, 100 g dry stems, leaves and flowers of *V. yedoensis* were soaked in 2.5 l of distilled water overnight and then boiled in water for 2 h. A crude dark brown water extract was obtained by filtering out the plant residue; 300 ml of the crude aqueous extract (residue dry weight: 3.67 g) was diluted with 1200 ml 95% ethanol (Sidebotham and Weigel, 1971). A copious quantity of gel like precipitate formed immediately. The alcohol extract mixture was divided equally into six 250 ml centrifuge bottles and subjected to centrifugation at 5900 × *g* (6000 r.p.m.; Sorvall RC-5B Refrigerated Superspeed Centrifuge with a GSA rotor) for 30 min at 15°C. The supernatant was recovered by filtration from the gel pellet. The gel pellet could not be readily redissolved in water, so a suspension of the pellet was prepared with shaking and designated as Fraction A. The supernatant solution recovered was evaporated to dryness (Buchi rotovapor) and then further dried in an oven under vacuum at about 90–100°C for about 2–3 min. This dried material was extracted with 700 ml methanol with stirring during 13 h. The resulting methanol extract was filtered from the remaining insoluble residue remaining in the flask. The insoluble residue was washed three times with 100 ml methanol and the methanol extracts were combined and evaporated to dryness (Buchi rotovapor) and dissolved in a minimum amount of water. Acetonitrile was added in excess (about 500 ml) until completion of precipitate formation. The fine dark brown precipitate was collected by vacuum filtration using a Magna Nylon 66 membrane filter paper (pore size 0.45 μm). Alternatively, the precipitate could be collected after centrifugation at 5900 × *g* for 30 min. The filtrate was refiltered twice and finally obtained as a clear yellow solution. It was evaporated to dryness and 0.227 g dry material was obtained which was then dissolved by shaking in 20 ml water. This solution was designated as Fraction B. The aforementioned dark brown precipitate was dissolved in water, filtered, evaporated to dryness and 0.940 g were isolated. This was dissolved in 90 ml water and designated as Fraction C.

The insoluble residue left after the 13 h of methanol extraction was further dried in a vacuum oven at around 90–100°C for 2–3 min. The resulting dry material was stirred with 200 ml acetone for 30 min, then dissolved in a minimum amount of water and diluted with about 250 ml acetonitrile. A dark brown precipitate was collected by vacuum filtration of the acetonitrile/water mixture using a membrane filter paper. The precipitate was washed three times with 50 ml acetonitrile and the filtrate was refiltered again, evaporated to dryness, and 5.38 mg material was obtained; this was dissolved in 1 ml water and designated as Fraction D. The dark brown precipitate was dissolved in water, evaporated to dryness (Buchi rotovapor) and further dried in a vacuum oven at around 90–100°C for 2–3 min. It was then washed with 100 ml tetrahydrofuran and 100 ml dimethylformamide, respectively,

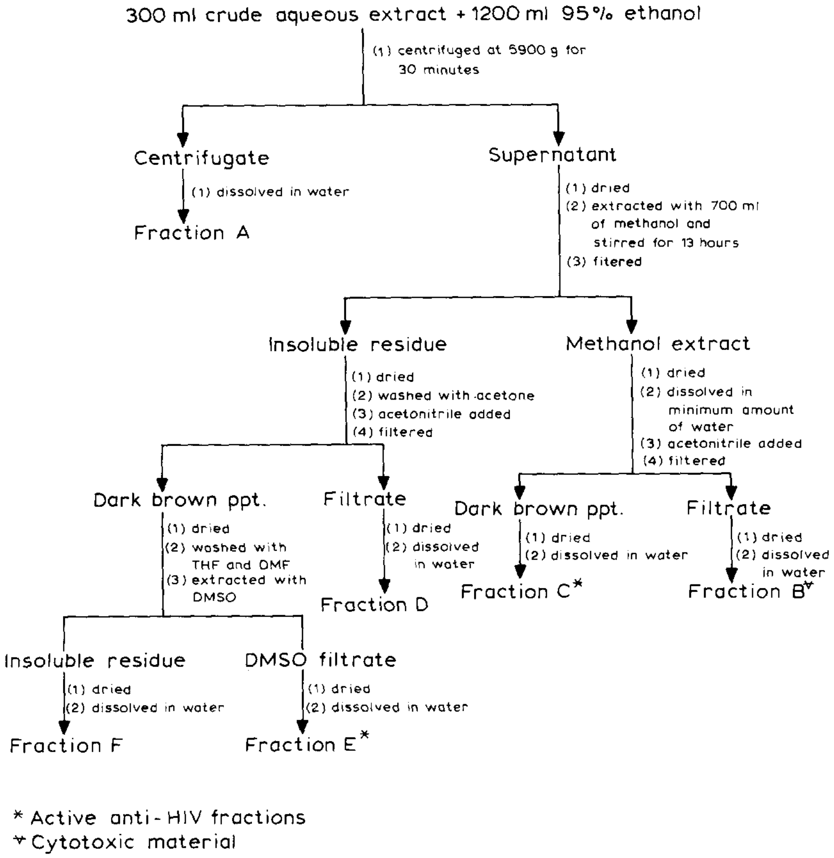

Fig. 1. Isolation and purification protocol for crude extract of *Viola yedoensis*.

and evaporation of these solvents yielded very little residue. The precipitate then was extracted, by shaking with 250 ml dry warm dimethylsulfoxide. The dark brown dimethylsulfoxide extract was filtered from a milky white residue through a membrane filter, was evaporated to dryness (rotary evaporator, 100°C, high vacuum) and the residue was washed successively with 50 ml acetone and 50 ml acetonitrile in order to remove residual dimethylsulfoxide, and then dried; 0.184 g material were isolated and dissolved in 18 ml water; this was designated as Fraction E. The above milky white residue was also dissolved in water, filtered, dried and 0.04 g material was obtained. It was dissolved in 4 ml water and designated as Fraction F. The entire isolation and purification flow chart is presented in Fig. 1.

HPLC

Fractions C and E (which showed anti-HIV activity, *vide infra*) were chromatographed by passing through a Bio-Sil TSK-250 HPLC gel filtration column (Kremmer and Boross, 1979) using a Waters Associates model 510 pump and usually a LDC/Milton Roy SpectroMonitor detector; alternatively, a refractometer was used for detection, and afforded identical results. Figs. 2 and 3 show the traces from each fraction. Fraction C in water was evaporated to dryness and extracted with dimethylsulfoxide. However, the material was totally soluble in dimethylsulfoxide and because of the complexity of its HPLC trace (Figure 2) it was not further studied. Only one peak was observed (Figure 3) from Fraction E.

Characterization of Fraction E

Fraction E (3.23 mg) was dissolved in D_2O and its 300 MHz proton NMR spectrum was measured using a General Electric QE300 spectrometer. The IR spectrum was measured, as a KBr disk, using an IBM FT/IR system 9000 instrument.

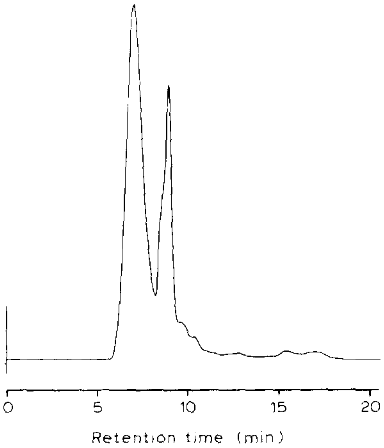

Fig. 2. Fraction C separation on analytical gel filtration HPLC column. Conditions: 50 μl injection, eluting with pure distilled water at room temperature and flow rate of 1 ml/min. Detector set at 299 nm.

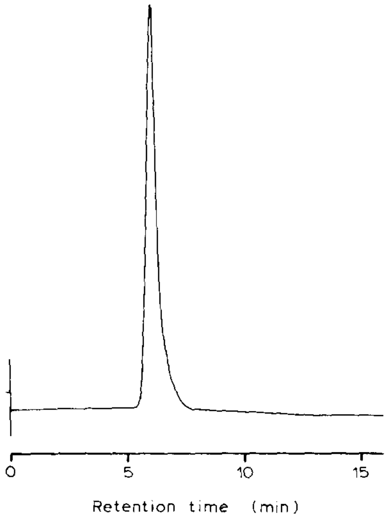

Fig. 3. Fraction E HPLC trace on analytical gel filtration column. Conditions as for Fig. 2.

The optical spectrum of Fraction E in water, measured using a Hewlett-Packard HP8450A diode array spectrophotometer, is shown in Fig. 4. The pH of Fraction E in water was also determined (Beckman model 3550 digital pH meter). The

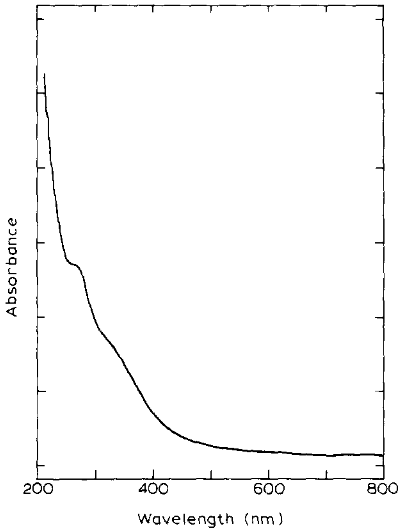

Fig. 4. Optical spectrum, in distilled water, of Fraction E.

complete Fraction E (Fig. 1; *vide supra*) was used in standard carbohydrate and protein tests (Shriner et al., 1980). Pure E was analyzed for sulfur at the Microchemical Analysis Laboratory, University of California, Berkeley.

Determination of molecular weight of Fraction E

A gel filtration standard (Bio-Rad) containing vitamin B_{12} (MW 1350), myoglobin (MW 17 000), ovalbumin (MW 44 000), IgG (MW 158 000), and thyroglobulin (MW 670 000) was passed through the analytical Bio-Sil TSK-250 gel filtration column using the same conditions as in separation of Fraction E, except the UV wavelength was changed to 280 nm and the eluant was 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4, pH 6.8. The standard was then spiked with Fraction E, and the HPLC was re-run under identical conditions (Chaplin and Kennedy, 1985). Fraction E eluted as a broad peak between the myoglobin and vitamin B_{12} peaks; Fig. 5 shows a plot of $\log_{10}$ of the molecular weight of the proteins versus their retention times, and indicates an MW of between 10 000 and 15 000 for Fraction E, which had a retention time of 14.0 min. The procedure was then internally calibrated by using a commercially available dextran of average MW 12 000 (from Spectrum Chemical Mfg. Corp.) to confirm its applicability to carbohydrate polymers. This procedure (dextran retention time 14.08 min) indicated that the 10 000 to 15 000 result for Fraction E (Fig. 5) was accurate. The molecular weight of Fraction E was also determined using Squire's method (Squire, 1964; Kosenko, 1983). Thus, Fraction E was mixed with Dextran Blue 2000 (Pharmacia, MW 2 000 000) and eluted through a Sephadex G-75 column using pure water elution. The maximum concentration

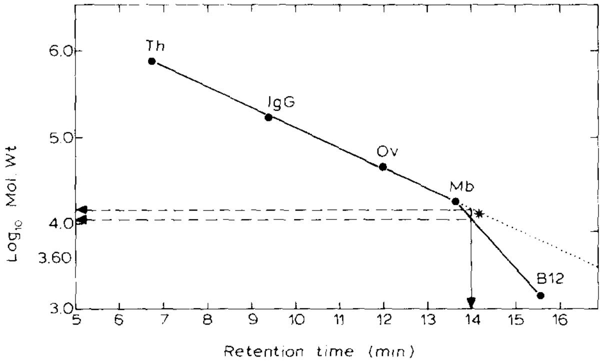

Fig. 5. Plot of $\log_{10}$ MW vs. HPLC retention time (mins) for a commercial standard mixture (**Th**, thyroglobulin, 670 000 daltons; **IgG**, gamma globulin, 158 000; **Ov**, ovalbumin, 44 000; **Mb**, myoglobin, 17 000; **B12**, Vitamin B_{12}, 1350). The column used is known to be inaccurate at very low molecular weights, hence the non-linearity with the standard mixture. Fraction E afforded a retention time of 14.0 min when spiked into this mixture, indicating an approximate MW 10 000 to 15 000. The asterisk indicates the commercial dextran of MW 12 000, with retention time 14.08 min, and enables calibration of the column for polysaccharides at this molecular weight. HPLC conditions: Bio-Sil TSK-250 HPLC gel filtration column using a Waters Associates model 510 pump and a LDC/Milton Roy Spectro-Monitor detector set at 280 nm; eluant was 0.1 M Na_2SO_4, 0.02 M NaH_2PO_4, pH 6.8, at room temperature and flow rate 1 ml/min.

of blue dextran appeared at a void volume of 84 ml, whereas the maximum concentration of Fraction E was measured at 151 ml. Application of these values in Squire's equation affords a calculated MW of 12000.

Testing for anti-HIV activity

Each fraction was titered for anti-HIV activity by determining the highest dilution which significantly reduced the percentage of HIV antigen-positive cells in the HIV-infected H9 cell culture. The procedure has been described in detail elsewhere (Chang and Yeung, 1988). Briefly, the fraction to be tested was diluted twofold serially in cell culture medium. To 0.2 ml diluted material, 0.7 ml H9 cell suspension (10^5 cells/ml) and 0.1 ml HIV ($10^{5.5}$ $TCID_{50}$/ml) were added. After 72 h, cells were smeared, fixed in acetone-methanol, stained for HIV antigens by immunofluorescence and scored for HIV-antigen positive cells. A 50% reduction or a reduction of 3 standard deviations (whichever is the larger reduction) from the mean of the untreated control was considered as significant reduction. Untreated control consisted of 0.2 ml medium, 0.7 ml H9 cells and 0.1 ml HIV in quadruplicate. The minimal inhibitory concentration is the least amount of the test material which achieves a significant reduction. For zidovudine, the minimal inhibitory concentration varied from 0.02 to 0.16 μg/ml (average 0.08), a value close to that ($ID_{90} \leqslant 0.13$ μg/ml) reported by the manufacturer.

Testing for cytotoxicity

Each fraction was titered for toxicity to the H9 cell (Popovic et al., 1984) the target cell for HIV by a standard procedure described in detail elsewhere (Chang and Yeung, 1988). The subtoxic concentration was the highest concentration which was nontoxic to the H9 cell.

TABLE 1

Subtoxic concentrations for the H9 cells and minimal inhibitory dose for the HIV of fractions from *V. yedoensis*

Fraction	Subtoxic concentration (μg/ml)	Minimal inhibitory dose (μg/ml)
Unfractionated	⩾ 200	200
A	50	Neg.[a]
B	13	Neg.[a]
C	⩾ 200	50
D	⩾ 200	Neg.[a]
E	⩾ 200	3
F	⩾ 200	Neg.[a]

[a] Non-inhibitory at subtoxic concentration.

Results and Discussion

After testing the anti-HIV activity and the cytotoxicity of each fraction from A to E, only fractions C and E were active against HIV as shown in Table 1. Fraction B was shown to contain the cytotoxic material. Fraction E has a minimal inhibitory concentration of 3 μg/ml, which was seventeen times more active than Fraction C. The minimal inhibitory concentrations of four other preparations of Fraction E varied from 3 to 16 μg/ml. In similar assays of dextran sulphate, the minimal inhibitory concentration varied from 0.4 to 1.6 μg/ml (average 1.0 μg/ml). Unfortunately, Fraction C could not be further purified at the present time owing to its complete solubility in dimethylsulfoxide, and the complexity of its HPLC profile (Fig. 2). The purity of the anti-HIV compound (Fraction E; Fig. 3) is established by high performance liquid chromatography gel filtration. The NMR spectrum of Fraction E, which had multiple peaks at chemical shifts in the range 3.2–5.0 ppm, indicated that it is a carbohydrate derivative (Pouchert, 1983). It showed no aromatic protons, indicating the absence of phenolic residues. The pH of fraction E in water was 6.53. Fraction E did not react in the Biuret test before acid hydrolysis (Shriner et al., 1980), indicating the absence of peptide fragments. Following complete hydrolysis in hot 10% aqueous HCl and neutralized by 10% NaOH, aliquots were negative in the ninhydrin test (for amino acids), but gave a positive Benedict's test for carbohydrate. On the basis of the retention time for the Fraction E material, (14.0 min; Fig. 5), the MW of the anti-HIV material is estimated to be between 10 000 and 15 000. These measurements were then calibrated by use of a known commercially available dextran standard of MW 12 000, which eluted at 14.08 min (indicated by an asterisk in Fig. 5); this calibration confirmed that the MW of Fraction E did indeed lie between 10 000 and 15 000. Finally, the MW of Fraction E was also determined using Sephadex G-75 gel filtration, and elution volumes of a standard and of Fraction E were substituted into Squire's equation (Squire, 1964) to afford a calculated MW of 12 000. This type of measurement is normally considered to be accurate to within 2–8% when the ratio of the elution volume of the unknown to the void volume of the column is $\leqslant 2.40$ (Kosenko, 1983). A combination of the various methods yields an MW value for Fraction E, the active anti-HIV component in *Viola yedoensis*, to be between 10 000 and 15 000. The pure compound is therefore a polysaccharide as indicated by its high molecular weight, water solubility, and NMR (HOD peak at 4.67 ppm) as well as IR data (broad OH stretching band between 3200 and 3600, 1632, 1400, 1078, 619 cm^{-1}). The 1400, 1078, and 619 cm^{-1} absorptions are characteristic (Socrates, 1980; Shriner et al., 1980) of an organic sulphonate salt (1420–1330, 1070–1030, 700–600 cm^{-1}). Preliminary paper chromatography studies of the acid hydrolysate indicate galactose to be the major sugar, along with a small amount of glucose. Elemental analysis of Fraction E for sulfur showed 4.85% sulfur content; on the basis of the infrared absorptions and the fact that, for example, suramin owes its anti-HIV activity to the presence of sulfonate groups (Balzarini et al., 1986), we assume the sulfur is present in the form of sulfonate, and this figure correlates with about one-half of a sulfonate residue per sugar (Ricketts, 1952).

Fractions C and E are dark brown in color (Fig. 4), this being associated with the anti-HIV activity, and thereby aiding its detection by spectrophotometry in HPLC . It was not possible to decolorize this fraction with activated charcoal without concomitantly losing the anti-HIV activity. The pure compound was quite heat stable since it could stand temperatures in excess of 100°C without loss in activity. However, it should be kept frozen in order to prevent bacterial or fungal growth.

The extraction procedure of *V. yedoensis* with dimethylsulfoxide is a crucial step. The crude extract has to be dried thoroughly before extraction with dry dimethylsulfoxide. Otherwise the HPLC trace shows multiple peaks indicating the presence of impurities.

The antiviral attributes of Fraction E at 100 μg/ml are similar to those of the crude extract at subtoxic concentrations (Chang and Yeung, 1988); it does not induce interferons, does not inactivate extracellular HIV or herpes simplex virus, and is inhibitory to the HIV but not herpes simplex virus.

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