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Inhibition of growth of human immunodeficiency virus in vitro by crude extracts of Chinese medicinal herbs

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Summary

Twenty-seven medicinal herbs reputed in ancient Chinese folklore to have anti-infective properties were extracted by boiling under reflux. The extracts were tested for inhibitory activity against the human immunodeficiency virus in the H9 cell line at concentrations nontoxic to growth of the H9 cells. Using a significant reduction (>3 S. D. below the mean) in the percentage of cells positive for specific viral antigens in three successive assays as indicative of activity against the virus, 11 of the 27 extracts were found to be active. One of the extracts (*Viola yedoensis*) was studied in greater depth. At a subtoxic concentration, this extract shut off completely the growth of HIV in virtually all experiments. It did not inactivate HIV extracellularly, did not induce interferon and did not inhibit the growth of herpes simplex, polio or vesicular stomatitis viruses in human fibroblast culture. Chinese medicinal herbs appeared to be a rich source of potentially useful materials for the treatment of human immunodeficiency virus infection.

HIV; Anti-HIV; Chinese medicinal herbs; AIDS

Introduction

Several compounds have been reported to be inhibitory to the growth of the human immunodeficiency virus (HIV) in vitro (Anand et al., 1986; Balzarini et al.,

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1986; Ho et al., 1985; McCormick et al., 1984; Mitchell et al., 1987; Mitsuya et al., 1984, 1985, 1986; Pert et al., 1986; Pizzi, 1950; Rozenbaum et al., 1985; Sandstrom et al., 1985; Veno and Kino, 1987; Yamamoto et al., 1986). However, these compounds are too toxic for prolonged use or are incapable of eliminating HIV infection from the human host (Blanche et al., 1986; De Clercq, 1986; Rozenbaum et al., 1985; Yarchoan et al., 1986; Wetterberg et al., 1987). There is an urgent need to continue the search for less toxic and more effective anti-HIV substances. To meet this need, we have initiated a long-termed study to evaluate herbal remedies reputed in ancient Chinese folklore to have anti-infective properties. This report describes results of our preliminary screening of 27 Chinese medicinal herbs for anti-HIV activities. One of the extracts (*Viola yedoensis*) was studied in greater depth; the results are also presented here.

Materials and Methods

Selection of herbs

With the aid of a computerized data base at the Chinese Medical Material Center of the Chinese University of Hong Kong, we identified a partial list of 27 herbs said to have anti-infective activities (Jiangxu New Medical College). These herbs are: *Houttuynia cordata, Prunella vulgaris, Lithospermum erythrorhizon, Alternanthera philoxeroides, Chrysanthemum morifolium, Arctium lappa L., Bupleurum chinense, Epimedium grandiflorum, Lonicera japonica, Woodwardia unigemmata, Lysimachia foenum, Viola yedoensis, Forsythia suspensa, Isatis tinctoria, Senecio scandens, Panax ginseng, Artemisia apiacea, Paeonia lactiflora, Andrographis paniculata, Coptis chinensis, Origanum vulgare, Pulsatilla chinensis, Brucea javanica, Sophora flavescens, Scutellaria baicalensis, Rheum palmatum,* and *Polygonum cuspidatum.* They were purchased from Chinese medicinal pharmacies in Hong Kong and authenticated in the Museum for Chinese Medicinal Herbs of the Chinese University of Hong Kong. The following herbs were also purchased from a Chinese medical pharmacy in San Francisco: - *A. lappa, L. japonica, V. yedoensis, A. paniculata, C. chinensis, P. vulgaris* and *L. erythrorhizon.*

Preparation of crude extracts

Ten grams of dried herb were cut into small pieces, soaked in 100 ml water at room temperature overnight and then boiled under reflux for 1.5 h. The extract was decanted and centrifuged at 500 $\times$ *g* for 10 min. The supernatant fluid was dialyzed (Spectra/Por 1 MWCO 6000–8000) against 200 times its volume of phosphate buffer saline (pH 7.2) for 2 h at 0°C with constant stirring. The fluid in the dialysis bag was filtered through a 0.45 μm membrane filter and stored at below − 10°C until tested. The extract was dialyzed to remove some of the cytotoxic factors. Unless specified otherwise, a single preparation of each herb was used in all experiments reported.

Preparation of H9 cell suspension

The H9 cell (Popovic et al., 1984) was a gift from Dr. Robert E. Gallo to Dr. Murray B. Gardner. On receipt from Dr. Gardner we tested the cell for mycoplasma contamination by a routine procedure (Brooks et al., 1981); it was free of such a contamination. The cell was grown in a medium consisting of 10% inactivated fetal calf serum in RPMI with 20 μg of gentamicin per ml.

Preparation of the HIV pool

HIV was also a gift from Dr. Robert E. Gallo to Dr. Murray B. Gardner. The virus was obtained from chronically infected H9 cells. We have also found the culture to be free of mycoplasma contamination (Brooks et al., 1981). The infected H9 cells were grown in bulk quantity and subjected to freeze-thawing to disrupt the cells. The suspension of disrupted cells was filtered through a 0.45 μm membrane filter, the filtrate divided into one ml portions and stored at −70°C until use. Once thawed, the unused portion was discarded. A single pool was used in all experiments. This pool had an infectivity titer of $10^{5.3}$ $TCID_{50}$ per ml as determined by terminal dilution.

Determination of subtoxic concentration of herbal extracts

A herbal extract was diluted two-fold serially in medium. To 0.2 ml of the diluted extract, 0.8 ml of a freshly prepared H9 cell suspension was added. This was done in duplicate. A medium control was included in every assay. This medium control consisted of 0.8 ml of the same H9 cell suspension added to 0.2 ml of medium. The control was done in quadruplicate. After 4 days of incubation, the number of viable cells in each culture was counted with a hemacytometer by dye exclusion (Merchant et al., 1960). When the viable count of extract-treated culture was 2 S. D. below the mean of the medium control, the extract-treated culture was considered to show evidence of cytotoxicity. The highest concentration of an extract which showed no evidence of cytotoxicity was taken as the subtoxic concentration of the extract.

Testing for anti-HIV activity

To 0.2 ml of medium containing 5 times the subtoxic concentration of an extract to be tested, 0.7 ml of H9 cell suspension and 0.1 ml of the HIV pool were added. This was done in duplicate in most assays. In each assay, a virus and a cell control were included. Both controls were done in quadruplicate. After 4 days of incubation, the cultures were given code numbers and cells in each culture smeared. Smears were fixed in acetone-methanol and stained for HIV antigen by indirect immunofluorescence (Kaminsky et al., 1985). The positive serum was a pool of sera from 18 homosexual males suffering from the lymphadenopathy syndrome. The serum pool had a titer of 1/320 against the HIV-infected H9 cells by indirect immunofluorescence. The negative serum was from a heterosexual male known to be negative for HIV antibody. Both sera were used at 1/10 in the indirect immunofluorescence test.

Smears were scored for cells with and without fluorescence. Cells with fluores-

cence on the membrane, in the cytoplasm or both were considered as fluorescent cells. At least 4 random fields (40 × objective) with confluent or near confluent cell monolayer were scored. The number of cells in each field was estimated to be 700. Total cell number in each field was first estimated under bright light illumination; and then fluorescent cells counted under ultraviolet illumination. In some smears without confluent or near confluent monolayer of cells, at least 500 cells were scored. A value of ≥ 3 S. D. below the mean of the virus control was considered as evidence for a significant reduction in the percentage of HIV-antigen positive cells. Percent inhibition was calculated by the following formula: 100 × (% positive cells in virus control − % positive cells in treated culture) divided by % positive cells in virus control.

Determining the infectivity of HIV in culture fluid

The culture to be tested was centrifuged at 3000 × *g* for 3 min in a Fisher centrifuge. The supernatant fluid was diluted 10-fold serially. One-tenth ml of diluted material was added to 0.2 ml of H9 suspension. Four cultures were so prepared for each dilution of test material. The culture volume was doubled every fourth day with fresh medium. On the 16th day, the cultures were examined for characteristic cytopathic change (large 'ballooned' multinucleated cells). Cells in cultures without cytopathic changes were smeared for HIV antigen testing by immunofluorescence. Cultures positive for cytopathic changes or HIV antigen were scored as positive for infectious HIV. The results were expressed as $TCID_{50}$ according to the formula of Reed and Muench (1938). Based on Pizzi's equation (Pizzi, 1950), a difference of ≥ 1 log $TCID_{50}$ between 2 preparations is statistically significant.

Testing extract for direct virocidal effect against HIV

To 0.1 ml of HIV, 0.7 ml of medium and 0.2 ml of the extract at 5 times subtoxic concentration was added. For control, 0.9 ml medium was added to 0.1 ml HIV. Both were incubated in a 37°C water bath for 60 min and then titrated for residual HIV infectivity.

Test for interferon induction

Standard procedures were used (Merigan, 1971; Spina et al., 1972). Briefly, 2 ml medium containing the extract at subtoxic concentration was added to a confluent culture of human fibroblasts in a 3.5 cm diameter Petri dish. After 20 h at 36°C, the medium was removed for interferon assay (Merigan, 1971) and the culture tested for susceptibility to vesicular stomatitis virus by plaque assay. Positive and negative controls were included in each assay. For positive control, poly I;C (B.L. Bio-Chemical) was used; and for negative control, plain medium was used. Fluid removed for interferon assay was generally stored at −70°C and tested at a later date by the 50% plaque-reduction test (Merigan, 1971).

Testing extract for inhibitory activity against the growth of herpes simplex virus type II (HSV), polio virus type I (PV) and vesicular stomatitis virus

The following two tests were used: the 50% plaque reduction (Richards et al., 1978) and the inhibition of cytopathic changes. All tests were performed with confluent monolayer cultures of human embryonic fibroblasts. In the 50% plaque reduction, about 20 plaque forming units of virus were added to each culture. After 30 min at 36°C, agar overlayer was added. The agar overlay for control cultures was medium with 1% agar; that for the treated culture, medium with 1% agar and the extract at subtoxic concentration. The cultures were scored for plaques 48 h later.

In the test for inhibition of cytopathic changes, we inoculated 3×10^5 fibroblasts in 0.7 ml medium into each of the 24 wells in a tissue culture cluster. After 20 h at 36°C, when the inoculated cells formed a confluent monolayer, 0.1 ml of virus containing about 20 plaque-forming units was added to each well. After 30 min, 0.2 ml medium was added to each virus control well and 0.2 ml medium containing the extract at 5 times the subtoxic concentration was added to each treated well. The extent of cytopathic changes in the virus control and in the treated wells was compared once every 24 h.

Results

Determination of subtoxic concentration

Subtoxic concentrations of herbal extracts to the H9 cell were reproducibly determined by the described method. Subtoxic concentrations for these herbal extracts varied from 1/10 to 1/640. The *V. yedoensis* extract was nontoxic at 1/40; at this concentration, the H9 increased by 11- to 15-fold in 96 h.

Scoring of HIV antigen-positive (fluorescent) cells

The scoring of fluorescent cells is generally known to be inaccurate. To illustrate the degree of variability, we summarize in Table 1 the results for virus control cultures in the 10 assays completed by early 1987. The percentages of HIV antigen-positive cells found in all virus control cultures varied from 8–40%; the mean and S. D. were 27 ± 10%. Variation within the same assay was considerably less; the largest difference between replicate was 2.5-fold in the 3rd assay. Our impression was that accurate scoring of smears with a large number of fluorescent cells (such as those found in the virus control) was difficult. But smears with less than 40 fluorescent cells per microscopic field (each field encompassed about 700 cells when the cells formed a confluent or near confluent monolayer) were easily and accurately scored. All smears from cell control cultures (H9 cells not infected by HIV) and all smears of HIV infected cells 'stained' with the negative serum were consistently scored as negative. These negative results attested to the high degree of specificity of the immunofluorescent test for the detection of HIV antigen (Kaminsky et al., 1985). These negative results were so consistent that we considered as superfluous the inclusion of these negative controls in the assay.

TABLE 1

Variations in the percentage of HIV antigen-positive cells as revealed by the percentages found in virus control cultures

Assay	% HIV antigen-positive cells in each culture*
1	13, 15, 8, 15 (13 ± 3)
2	37, 49, 28, 38 (38 ± 7)
3	21, 30, 12, 23 (22 ± 6)
4	42, 43, 41, 35 (40 ± 3)
5	18, 27, 29, 13 (22 ± 4)
6	17, 26, 30, 31 (26 ± 6)
7	28, 22, 25, 18 (23 ± 4)
8	24, 18, 20, 24 (22 ± 3)
9	22, 18, 25, 16 (20 ± 4)
10	36, 39, 36, 47 (40 ± 5)

*These cultures were 'virus control' (0.1 ml of HIV, 0.7 ml of H9 suspension and 0.2 ml medium); values in brackets are means and S. D.

Inhibition by selected herbal extracts of the growth of HIV in H9 cells

All the 27 herbal extracts were tested at subtoxic concentrations for anti-HIV activity. Eleven consistently reduced the percentage of HIV antigen-positive cells by 3 or more S. D. below the mean of the virus control (See Table 2). Extracts of *A. lappa L, V. yedoensis, A. paniculata, L. erythrorhizon* and *A. philoxeroides* virtually shut off all synthesis of HIV antigens as determined by indirect immunofluorescence. Others such as extracts of *W. unigemmata* and *S. scandens* yielded somewhat variable results. Seven extracts reduced the percentage of HIV antigen-positive cells by 3 or more S. D. in only 1 or 2 of the 3 assays; these are not listed in the Table as effective against the HIV. In our assay system, the minimal inhib-

TABLE 2

Reduction in percentage in HIV antigen-positive cells by herbal extracts at subtoxic concentrations

Culture treated with extract of:	% of HIV antigen-positive cells		
	Assay 1	Assay 2	Assay 3
None*	12.8 ± 2.9	38.4 ± 7.2	21.5 ± 6.4
A. lappa L	0	1	0
E. grandiflorum	0.1	0.2	3
L. japonica	0	2	2
W. unigemmata	0	6	3
V. yedoensis	0	0.4	0.4
S. scandens	0.5	9	2
A. paniculata	0	0.2	0
C. chinensis	0	0.2	4
P. vulgaris	0	1	3
L. erythrorhizon	0	0	0
A. philoxeroides	0	1	0

*Virus control (HIV-infected culture not treated with herbal extracts; in quadruplicate)

TABLE 3

Relation between percentage of HIV antigen-positive cells and infectivity titer

Culture treated with[1]	expt. 1		expt. 2	
	% Positive cells	$TCID_{50}$	% Positive cells	$TCID_{50}$
Virus control	39.5	4.5[2]	40.2	5.5
V. yedoensis	0	<0.5	0.1	1.0
L. japonica	0.4	0.8		
P. vulgaris	0	<0.5		
L. erythrorhizon	0.3	<0.5		
poly rI:rC			1.4	3.0

[1]All extracts were tested at the subtoxic concentration and poly rI:rC, at 100 μg per ml.
[2]log $TCID_{50}$.

itory concentration for Zidovudine was about 0.2 μg/ml; this concentration is close to that ($ID_{90} \leq 0.13$ μg/ml) reported by the manufacturer.

To determine if herbal extracts from another source might have similar anti-HIV activity, we attempted to procure the herbs listed in Table 2 from a Chinese medicinal herb store in San Francisco; all but *E. grandiflorum*, *W. unigemmata*, *S. scandens* and *A. philoxeroides* were available. Extracts were prepared as described from herbs purchased in San Francisco. All except *C. chinensis* were active against the HIV similar to herbs procured in Hong Kong.

Relation between percentage of HIV antigen-positive cells and infectivity titer

To determine the relation between percentage of HIV antigen-positive cells and infectivity titer, we made parallel determinations of percentage of antigen-positive cells and $TCID_{50}$ in culture fluid of several infected cultures. Results are summarized in Table 3. In general, a culture with high percentage of HIV antigen-positive cells also had high $TCID_{50}$. Conversely, low percentage of antigen-positive cells was associated with low $TCID_{50}$.

Relation between percent inhibition and time of adding extract (V. yedoensis)

To determine if *V. yedoensis* extract at subtoxic concentration was still effective against the HIV after the cell had been infected by the virus, we first mixed the cell with the virus and then added the extract at 3 time intervals (immediately and

TABLE 4

Percent inhibition of growth of HIV in H9 cells by *V. yedoensis* extract added at zero, 30 min and 4-h after infection

Expt.	% inhibition when extract was added at:		
	0 h	30 min	4 h
1	100	100	74
2	100	98	68
3	100	96	84

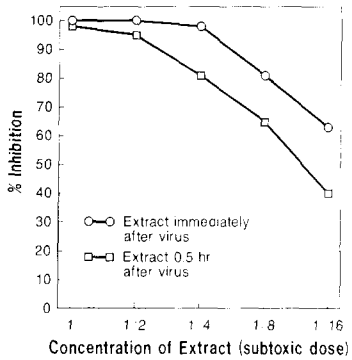

Fig. 1. Relation between percent inhibition of HIV in H9 cell cultures treated with *V. yedoensis* extract at subtoxic doses and fractions of subtoxic doses.

at 0.5 and 4 h). Results are summarized in Table 4. When the extract was added at a subtoxic concentration to the culture immediately after the addition of virus, the shutdown of HIV replication was complete (100% inhibition). With a delay of 0.5 h in treating the infected culture, the inhibition was less complete but still impressive (96–100% inhibition). A further delay to 4 h in treating the infected culture further reduced the effectiveness to 68–84% inhibition.

Relation between percent inhibition and concentration of extract (V. yedoensis)

Data from 2 such studies are illustrated in Fig. 1. There was a positive relation between the concentration of extract and percent inhibition of HIV growth.

V. yedoensis extract not virucidal to HIV

Exposure of the HIV to *V. yedoensis* extract at the final concentration of 1/40 (subtoxic conc) for 1 h at 37°C did not result in a significant drop in infectivity titer. The differences in $TCID_{50}$ (in log units) of the treated and control in three successive experiments were 0.5, − 0.4 and zero.

TABLE 5

Failure of *V. yedoensis* extract to induce resistance to vesicular stomatitis virus in human fibroblasts

Inducer	No. of VSV plaques* per culture		
	expt. 1	2	3
Medium control	28 ± 4	36 ± 4	36 ± 3
V. yedoensis extract	30	29	25
poly I:C 10 μg/ml	0	0	0
poly I:C 1 μg/ml	1	NT	NT
poly I:C 0.1 μg/ml	18	NT	NT

*All values were averages of duplicate cultures, except for medium controls which were averages of quadruplicates. NT = not tested.

Human fibroblasts or H9 cells not resistant to VSV after prior treatment with V. yedoensis extract

Human fibroblast cultures after 24 h exposure to *V. yedoensis* extract at subtoxic concentration at 36°C retained their susceptibility to VSV much like control cultures. In contrast, cultures treated with poly rI:rC at 10 μg per ml became resistant to the growth of VSV (Table 5). As expected, interferon activity was not detected in the medium of cultures treated with the extract (data not shown).

The extract at subtoxic concentration also failed to induce resistance to VSV in the H9 cells. The extract delayed neither the onset nor the progression of cytopathic changes. The yield of VSV in culture treated or not treated with the extract was not significantly different in the three experiments (data not shown).

Failure of V. yedoensis extract to inhibit the growth of herpes simplex, polio or vesicular stomatitis virus

The extracts at the subtoxic concentration of 1/40 were not inhibitory to HSV, PV or VSV in the 50% plaque reduction test. Similarly, in the test for inhibition of cytopathic changes, the extract at 1/40 dilution failed to delay the onset or reduce the extent of cytopathic changes induced by the three viruses (data not shown).

Discussion

We have used a relatively simple procedure to screen natural products for anti-HIV activity. This procedure requires minimal handling of infected cultures and utilizes facilities ordinarily found in a standard medical virology laboratory. One drawback of this procedure is the difficulty in accurately scoring the fluorescence-positive (HIV-positive) cells. Our data in Table 1 show that variations in the percentages of HIV antigen-positive cells in the 40 replicate cultures can be considerable, especially those in different assays. However, by comparing only data within the same assay, this variation is reduced. By scoring the cell smears under code, by assigning trained personnel to do the scoring, by regularly including virus control in quadruplicate, and by accepting as significant only those reductions of at least 3 S.D. below the mean of the virus control, it has been possible to identify reproducibly selected herbal extracts as inhibitory to the growth of HIV.

That the percentage of HIV antigen-positive cells in a culture is a measurement of the amount of infectious HIV synthesized by the culture is demonstrated by data in Table 3. Generally, there is a good positive relation between the percentage of antigen-positive cells and infectivity titer under our experimental conditions. This finding is consistent with those reported by others (Harada et al., 1985; Vogt et al., 1987).

Using the criterion of a reduction of HIV antigen-positive cells by 3 or more S. D. in 3 successive assays as indicative of anti-HIV activity, we have identified as anti-HIV 11 extracts from a list of 27 Chinese medicinal herbs reputed in ancient Chinese folklore to have anti-infective properties. Extracts of *A. lappa* L. *V. yedoensis, A. paniculata, L. erythrorhizon* and *A philoxeroides* at concentrations non-

toxic to the host cell (H9), achieved a 97–100% inhibition of the growth of HIV in vitro. Others extracts (*E. grandiflorum, L. japonica, W. unigemmata, S. scandens, C. chinensis,* and *P. vulgaris*), though meeting our arbitrary criteria for effective anti-HIV extract, gave somewhat variable results. For example, the extract of *S. scandens* caused a 96% inhibition in assay no. 1 but only a 77% inhibition in assay no. 2. The basis for this variation requires further study. Seven other extracts were inhibitory in only 1 or 2 of the required 3 consecutive assays, and are not listed in Table 3 as active against the HIV. It is possible that some of these inactive extracts may have weak anti-HIV activity.

For a country not known to have endogeneous HIV infection (Chang et al., 1986; Guo et al., 1986) it is surprising to find so many effective anti-HIV herbal extracts among her folk remedies. It is possible that many of the extracts contain a common substance active against the HIV. It is also possible that these antiviral substances are compounds with broad antiviral spectrum such as interferon-inducers or tannic acid related compounds (Breinig and Morahan, 1980; Kucera and Hermann, 1967; May and Willuhn, 1978; Van den Berghe et al., 1978). Higher plants and their products such as tea, caffeine, grapes, mint and many others (Hermann and Kucera, 1967; John and Mukundan, 1979; Konowalchuk and Spiers, 1976, 1978; Kucera and Hermann, 1967; May and Willuhn, 1978) are known to contain compounds with broad anti-viral activity spectrum. These compounds are believed to be tannins or related compounds which inactivate a variety of virions extracellularly. Interferon-inducers are sometimes found among natural products (Stewart, 1981) including Chinese medicinal herbs (Hou, 1983). If this speculation is correct, it is probably useless to study further whether these herbal extracts are useful in the therapy of AIDS. There is no conclusive evidence that interferon inducers are effective against AIDS; neither is there evidence that tannic acid or related compounds are effective anti-virals in vivo. Because of the practical importance of this speculative question, we have embarked on a more detailed study on extract of *V. yedoensis.*

Our data on *V. yedoensis* extract established conclusively that the extract at subtoxic concentration did not induce interferon. The extract did not inactivate extracellular HIV or inhibit the growth of herpes simplex, polio or vesicular stomatitis virus. Without chemical data, it is not possible to conclude that the anti-HIV component in *V. yedoensis* extract is not a tannin-like compound. Because of its failure to inactivate extracellular HIV and to inhibit the growth of herpes simplex, polio and vesicular stomatitis virus, we concluded that the anti-HIV component in *V. yedoensis* extract is dissimilar to the tannins in tea, grapes and mint.

Chinese folk medicine is based largely on anecdotal observations over the past several thousands of years. The effectiveness of therapeutic agents (mostly herbs) used by practitioners has seldom been proven by using adequate controls. It is quite possible that some herbal remedies may have specific therapeutic action as was proven to be the case with the anti-malarial, qinghaosu (Klayman, 1985). What is badly needed are data collected by modern scientific methods for the purpose of documenting the effectiveness of herbal remedies in the treatment of specific diseases. Taking this approach we have identified 11 Chinese medicinal herbs as ef-

fective inhibitors of the growth of HIV in vitro. With one of these herbs (*V. yedoensis)*, we have shown that it has no interferon-inducing capacity, it did not inactivate extracellular HIV and had a narrow antiviral activity spectrum. We believe that further evaluation of *V. yedoensis* and perhaps other herbs as anti-HIV drugs is indicated. The next phase of this evaluation process is the purification and identification of the anti-HIV compounds and the determination of its relationship to glycyrrhizin. Glycyrrhizin is an anti-HIV compound extracted from the Chinese medicinal herb, *Glycyrrhiza radix* (Ito et al., 1987). Since this phase of research requires expertise in organic chemistry of natural products, and since it is impractical for our two laboratories, separated by the Pacific Ocean, to further collaborate in this study, and since the senior author does not have this expertise, it appears justifiable to publish our research results in its present stage of development. Scientists interested in the anti-HIV activity of Chinese medicinal herbs may find our data useful.

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