응용약물학회지, The Journal of Applied Pharmacology 3, 210-216(1995)

Inhibition of HIV-1 Replication by the Water-soluble Extract Mixture of Ricini Semen and Coptidis Rhizoma

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(Received August 9, 1995; accepted August 29, 1995)

Abstract—Partially purified water-soluble extract mixture from Ricini and Coptidis (named as RIC) showed to be a potent inhibitor of human immunodeficiency virus-1 (HIV-1) replication. RIC was evaluated for *in vitro* anti-HIV activity using SupT1 and H9 cells infected by a recombinant virus (pSVCAT) containing chloramphenicol acetyltransferase (CAT) gene substituted for nef gene in the HIV-1 genome. RIC inhibited syncytia formation of SupT1 cells with a half maximal effective concentration, IC_{50}, of 2.5 $\mu g/ml$ and showed marked inhibition of CAT activity in the infected H9 cells and also suppressed reverse transcriptase (RT) activity in the supernatant of the infected H9 culture. However, RIC did not inhibit the activity of reverse transcriptase directly when it was mixed with the enzyme or with viral particles. Berberine, one of components of RIC, also showed similar anti-HIV activity as RIC did. The data suggest that there are active ingredients which mediate anti-HIV activity in RIC.

Keywords □ HIV-1, Ricini, Coptidis, anti-HIV activity, reverse transcriptase

Acquired immune deficiency syndrome (AIDS) is caused by the human immunodeficiency virus which is transmitted primarily by exposure to blood and blood products, sexual contact and from mother to child during the prenatal period (Gallo *et al.*, 1984).

Various strategies against AIDS have been tested and a number of compounds have been designed to develop anti-HIV agents (Johnston and Hoth, 1993). Several groups have tried to develop HIV specific protease inhibitors. They have tested a series of peptide derivatives for HIV protease inhibitors and found some derivatives which have antiviral activity (McQuade *et al.*, 1990; Roberts *et al.*, 1990; Meek *et al.*, 1990).

For the replication of HIV, conversion of RNA to complementary DNA by reverse transcriptase is essential. So many studies are focused on the development of reverse transcriptase inhibitor. The results with 3'-azido-3'-deoxythymidine (Zidovudin, AZT) as an inhibitor of reverse transcriptase are encouraging in indicating both increased survival and quality of life in treated patients (Fischl *et al.*, 1987). However, patients receiving AZT have displayed toxicity including headache, nausea, and fever (Richman *et al.*, 1987) and appearance of drug-resistant variants of HIV-1 on long-term treatment of these anti-HIV agents including AZT (Larder *et al.*, 1989; De Clercq, 1994). Many other investigators have designed nucleoside analogues to block reverse transcriptase activity. These studies have led to clinical application of 2',3'-dideoxycytosine (DDC), 2',3'-dideoxyadenosine (DDA), 2',3'-dideoxyinosine (DDI) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T). In addition to nucleoside analogues, nonnucleosidal inhibitor, tetrahydro-benzodiazepine (TIBO) have been investigated (Pauwels *et al.*, 1990; Spence *et al.*, 1995) to have inhibitory effect on HIV-1 reverse transcriptase.

Phosphate-methylated DNAs targeted at the transactivator responsive region and the primer binding site in the HIV-1 genome inhibit the viral infectivity (Buck *et al.*, 1990). Monoclonal antibodies against N- and O-linked carbohydrated epitopes, gp120, have shown to block viral infection and syncytium formation (Hansen *et al.*, 1990). More recently, compound Ro 5-3335 (7-chloro-5-(2-pyrryl)-3H-1,4-benzodiazepine-2(H)-one) which is designed to block the transactivator Tat has inhibitory effect on HIV replication (Hsu *et al.*, 1991).

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In addition to the above agents, oligonucleotides (Goodchild *et al.*, 1988; Sarin *et al.*, 1988), GLQ223 (McGrath *et al.*, 1989), dextran sulfate (Mitsuya *et al.*, 1988), soluble CD4 (Lifson *et al.*, 1988), D-penicillamine (Chandra *et al.*, 1986), and phosphonoformate (Sarin *et al.*, 1985) are demonstrated antiviral activity.

Other groups have reported that some natural products and their related compounds including hypericin (Merulelo *et al.*, 1988), amphotericin B (Scharffner *et al.*, 1986), N-butyldeoxynojirimycin (Karpas *et al.*, 1988), chalcones and flavones (Hatano *et al.*, 1988), pepstatin A (von der Helm *et al.*, 1989), and benanomicines A and B (Hoshino *et al.*, 1989) showed the anti-HIV activity.

However, effective and safe agents for AIDS have not found yet. Therefore, there still remains an urgent need to search for new inhibitors of HIV that could be useful in the treatment of AIDS patients. We have screened natural products which are being used traditionally for oriental medicine. These compounds have advantage regarding toxicity because they are proven not to have severe toxicity.

We tested the anti-HIV activity of various natural products including RIC, the water soluble extract of Ricini and Coptidis mixture, and AQD, the water soluble extract of Akebia and Crotonis mixture. This report demonstrates possible candidate among natural products, which have anti-HIV activity.

Materials and Methods

Materials

Ricini Semen, Coptidis Rhizoma, Akebiae Caulis and Crotonis Semen were obtained from local oriental medicine herb store. RPMI 1640 and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, USA). Fetal calf serum were obtained from HyClone Laboratories (Logan, UT, USA). AZT was obtained from Burrough-Wellcome Inc. (Research Triangle, NC, USA). [^{3}H]Thymidine trisphosphate (TTP), and [^{14}C] chloramphenicol were obtained from NEN (Boston, MA, USA). Berberine, acetyl CoA, trichloroacetic acid (TCA), polyethylene glycol, dithiothreitol, trypan blue, Triton X-100, thin layer chromatography (TLC) plate and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of RIC

Defatted starch of 200 g Ricini Semen and 200 g Coptidis Rhizoma were mixed and extracted with chloroform at 25℃ during 25 hours. We removed the organic solvent by filtration. Dried precipitants were extracted with 5 liters of distilled water at 100℃ during 3~4 hours. The resulting aqueous extract was concentrated under reduced pressure to 1500 m*l* and filtrated. Then the remaining water-soluble extract were partitioned with hexane and chloroform, sequentially. The extract was further purified with talc adsorption, membrane filtration, and lyophilization to yield approximately 40 g of yellowish brown powder.

Preparation of AQD

200 g Akebiae Caulis and 200 g Crotonis Semen were mixed and extracted using the same scheme for RIC.

Virus and cells

HIV-1 and recombinant virus (pSVCAT) which has chloramphenicol acetyltransferase (CAT) gene substituted for nef gene and T cell lines such as SupT1 and H9 were kindly supplied from Dr. Y. C. Sung (POSTECH). We used SupT1 cells for syncytium formation assay and used H9 cells for the assays of reverse transcriptase (RT) and CAT activities. H9 and SupT1 cells were maintained at 37℃ in RPMI 1640 supplemented with 10%(v/v) of heat-inactivated fetal calf serum and 1%(v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2.

Toxicity assay

Toxicity of the RIC was examined by incubating 5×10^5 SupT1 cells in 24-well culture plates in growth medium containing variable concentrations of compounds. Live-cell counts were obtained from inoculation day to day 4 using trypan blue exclusion. Each data points represent the average values counted from two wells.

Syncytium formation assay

In 96-well plate, 5×10^4 SupT1 cells were challenged with 50 tissue culture infected dose ($TCID_{50}$) pSVCAT per well in the presence of variable concentrations of drugs and incubated for 3 to 4 days. The number of syncytia were counted by examining with inverted microscope. Data represent the average number of syncytia counted under the observation with inverted microscope.

Reverse transcriptase (RT) assay

For this purpose, 2.5×10^5 H9 cells were infected by pSVCAT in the absence or presence of compound in 24-well plate. In every third day, 1/4 of cultures were transferred to new media containing the same concentration of the compound. The cultures were harvested after 9 days and centrifuged for 3 minutes in Eppendorf centrifuge. The 600 μl of supernatant was mixed with 300 μl of 30% polyethylene glycol (PEG) and then centrifuged for 10 minutes. The pellet was

resuspended in RT suspension buffer (50 mM Tris, 1 mM dithiothreitol, 20% glycerol, 0.25 M KCl, 0.25% Triton X-100, pH 7.5). These virus samples were frozen at −70℃ until assayed. Aliquots of virus (10 μl were incubated with 50 μl of assay buffer (50 mM Tris, 5 mM dithiothreitol, 5 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.3 mM glutathione, 0.5 mM EGTA, 50 μg/ml poly(rA)dT, 10 μCi [^{3}H]TTP, and 17.25 μl distilled water, pH 7.5) for 1 hour at 37℃ (Hoffman *et al.*, 1985). Reactions were stopped by placing the tubes on ice and adding 2 ml of ice-cold pyrophosphate (0.01 M in 1 N HCl) and 2 ml of ice-cold 10% trichloroacetic acid (TCA). Precipitates were collected on Whatman GF/C glass fibers, washed several times with ice-cold 5% TCA and once with 70% ethanol, air dried and counted in a liquid scintillation counter. Duplicate experiments were carried out and the average values were presented.

Chloramphenicol acetyltransferase (CAT) assay

H9 cells infected with pSVCAT for reverse transcriptase assay were harvested and the cell pellet was used for CAT assay. The cell pellets were resuspended in 100 μl of 20 mM Tris (pH 8.0) and heat-inactivated at 60℃ for 30 minutes, and then frozen and thawed three times to lyse the cells. The cell lysates were centrifuged in microcentrifuge with 12,000 rpm at 4℃ for 10 minutes. The 70 μl of supernatants were transferred to 10 μl of reaction mixture (60 μl [^{14}C]chloramphenicol, 8 mg acetyl CoA, 540 μl distilled water) and incubated at 37℃ for 15 minutes and then stopped the reaction by addition of 1 ml ethyl acetate. The mixture was spin down for 2 minutes and 0.9 ml of the upper organic layer was dried for 40 minutes in Speed Vac to evaporate ethyl acetate and then dissolved in 20 μl of fresh ethyl acetate. The acetylated chloramphenicol was separated by thin layer chromatography (TLC) and analysed by exposure of TLC plate to X-ray film overnight.

Composition analysis

Total protein content was determined by Kjeldahl nitrogen analysis method (Harris, 1991). The contents of reducing sugar and crude ash were determined by the method described by Isaac (1990). In HPLC (Waters 510, U.S.A.) analysis, we used Bondapak C_{18} column (3.0×300 mm, Waters, U.S.A.) and UV detector at 280 nm. Mobile phase was sovent mixture of acetonitrile, 1.15 M NaH_2PO_4, and sodium dodecyl sulfate (45 : 55 : 0.2, v/v/w), and flow rate was 0.5 ml/min.

Results

Cytotoxicity of RIC

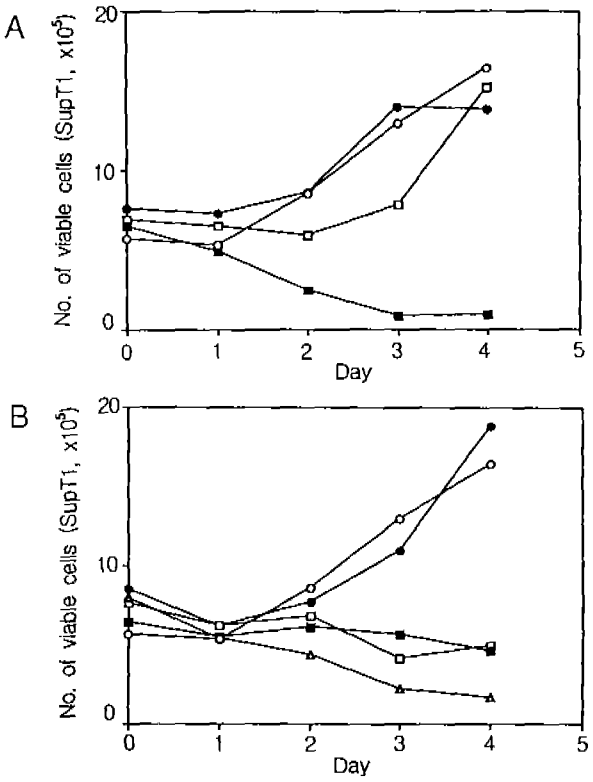

Fig. 1. Cytotoxicity of RIC and AQD in the supT1 culture. A, About 5×10^5 cells were plated in the 24-well plate and cultured in the absence (open circle) or presence of 100 μg/ml (closed circle), 200 μg/ml (open square), and 500 μg/ml (closed square) AQD for the indictated periods of time. B, Cells were counted after incubation with various concentrations of RIC; control (open circle), 10 μg/ml (closed circle), 50 μg/ml (open square), 100 μg/ml (closed square), 500 μg/ml (triangle). Four independent experiments were carried out and the results were reproducible.

The T lymphocyte cell line, SupT1 was cultured in the various concentrations of drugs and the number of viable cells was counted by hemocytometer. RIC had greater cytotoxic effect compared to AQD. AQD shows cytotoxicity at 500 μg/ml (Fig. 1A). As shown in Fig. 1B, RIC almost had no toxicity at 10 μg/ml, but showed toxicity above 50 μg/ml. So we further analyzed various effects of these herb extracts on HIV replication using the concentration range which did not have cytotoxicity.

Inhibitory effect of RIC on the formation of syncytia

SupT1 cells infected with pSVCAT formed syncytia after 3~4 days. We tested the effect of drugs including RIC and AQD on the formation of syncytia. The cells formed syncytia which can be identified easily by the observation with inverted microscope (Fig. 2). RIC showed inhibitory effect of syncytia formation with a half maximal inhibitory concentration (IC_{50}) approximately 2.5 μg/ml whereas AQD did not inhibit syncytia

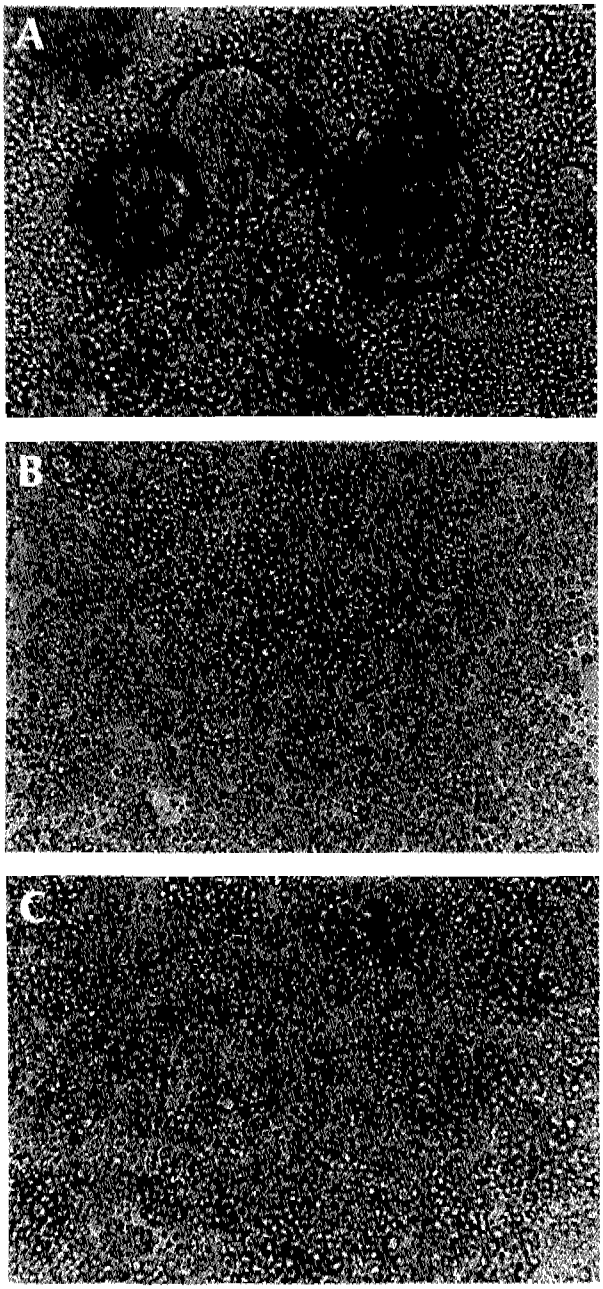

Fig. 2. Inhibition of syncytia formation in pSVCAT-infected SupT1 cell by RIC and berberine. The cells were challenged with pSVCAT (50 $TCID_{50}$) and incubated for 3 to 4 days in the presence of drugs. The formation of syncytia were observed with inverted microscope. A, control; B, RIC 10 $\mu g/ml$; C, berberine 3 $\mu g/ml$.

formation (Table I). We used AZT as a positive control in the inhibition of syncytia formation. AZT also inhibited syncytia formation very effectively. Berberine, one of major component of RIC, had inhibitory activity with an IC_{50} approximately 0.5 $\mu g/ml$. The result indicates that berberine is one of active components which have anti-HIV activity. The effects of drugs were reproducible in SupT1 cells infected with wild type HIV-1.

Table I. Drug effects on syncytia formation and cytotoxic effect

Drug	Concentration	Number of syncytia	Cytotoxic Effect
AQD	0 $\mu g/ml$	7	−
	10	2	−
	50	4	−
	100	5	−
	200	5	−
RIC	0 $\mu g/ml$	9	−
	2.5	3	−
	10	0	−
	50	0	+++
	100	0	++++
Berberine	0 $\mu g/ml$	9	−
	0.1	8	−
	0.5	5	−
	1.0	2	−
	3.0	1	+
AZT (Zidovudin)	0 μM	8	−
	0.05	1	−
	0.2	0	−
	1.0	0	−
	5.0	0	−

SupT1 cells (5×10^4 cells /well) were challenged with pSVCAT (50 $TCID_{50}$/well) in the presence of indicated compounds. Syncytia were counted by the observation with inverted microscope after 3 days incubation. No cytotoxic effect (−) were observed; cells counted less than 10% (+), over 50% (+++), over 80% (++++) of the population were shown to have cytotoxicity after 3 days incubation with indicated concentrations of drugs. The result is the representative of eight separate experiments that the results were reproducible.

Reverse transcriptase (RT) activity

Fig. 3 depicts reverse transcriptase activities obtained after 9 days cultures of pSVCAT-infected H9 cells in the presence of tested compounds. RIC 2.0 $\mu g/ml$ showed significant inhibition of viral RT activity and berberine 0.5 $\mu g/ml$ also had marked inhibitory effect on RT activity. AZT 0.2 μM as a positive control had comparable activity with the above concentrations of RIC and berberine. We further tested whether RIC and berberine inhibit RT directly. We mixed viral particles with these compounds and then checked the activity of RT. As shown in Fig. 4, RIC or berberine did not inhibit viral RT activity with a direct mode as AZT did not inhibit RT directly. These results suggest that RIC and berberine may not interrupt the action of RT in the process of viral replication.

Inhibition of chloramphenicol acetyltransferase (CAT) activity by RIC and berberine

pSVCAT contains CAT gene in the region of nef

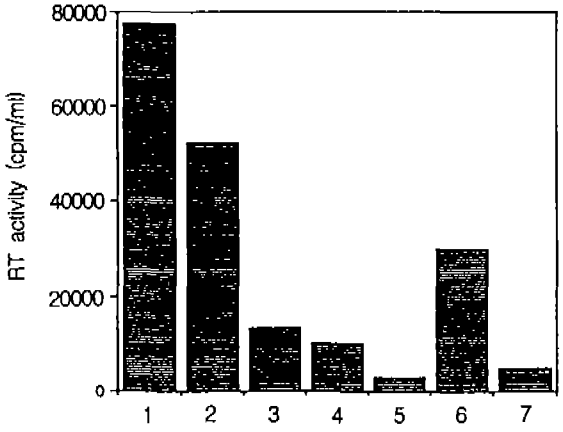

Fig. 3. Inhibition of reverse transcriptase activity by RIC and berberine. Reverse transcriptase activities in the pSV-CAT-infected H9 cell cultures were measured after incubation with various concentrations of compounds. 1, control; 2, AZT 0.05 M; 3, AZT 0.2 M; 4, RIC 2 μg/ml; 5, RIC 5 μg/ml; 6, berberine 0.1 μg/ml; 7, berberine 0.5 μg/ml. The result is the representative of three separate experiments and the results were reproducible.

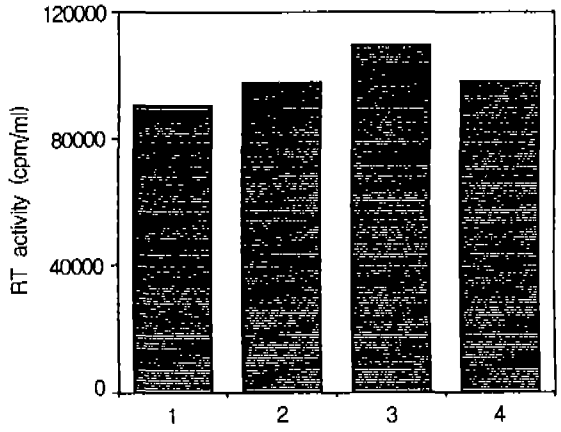

Fig. 4. Lack of direct inhibition of reverse transcriptase activity by RIC or berberine. Viral particles were mixed with various compounds and reverse transcriptase activities were measured. Direct inhibition of reverse transcriptase was not found by any compounds. 1, control; 2, AZT 0.2 M; 3, berberine 2.0 μg/ml; 4, RIC 10 μg/ml. Three seperate experiments were carried out and the results were reproducible.

gene in the HIV-1 genome. So CAT can be expressed during viral replication. We measured the activity of CAT to manifest the inhibitory effect of RIC and berberine on viral replication. CAT activity was markedly decreased in the pSVCAT-infected H9 cell culture in the presence of RIC or berberine (Fig. 5). RIC 2.0 μg/ml and berberine 0.5 μg/ml had better activity than 0.2 μM of AZT in the inhibition of CAT activity. AQD, a negative control, did not have inhibitory activity on CAT activity. The data also indicate that RIC and berberine effectively inhibit viral replication.

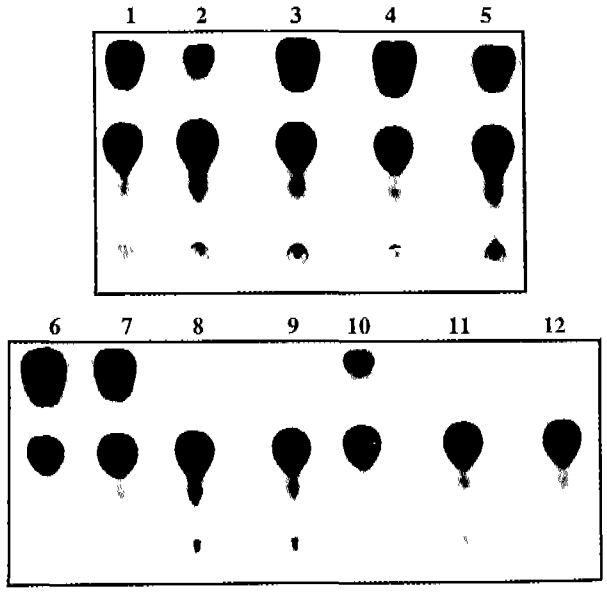

Fig. 5. Inhibition of chloramphenicol acetyltransferase (CAT) activity by RIC and berberine. CAT activities in the pSVCAT-infected H9 cells were measured after 9-day incubation with various compounds. 1, AZT 0.05 M; 2, AZT 0.2 M; 3, control; 4, ABBOTT protease inhibitor 0.05 M; 5, ABBOTT protease inhibitor 0.2 M; 6, AQD 10 μg/ml; 7, AQD 100 μg/ml; 8, RIC 2.0 μg/ml; 9, RIC 5.0 μg/ml; 10, berberine 0.1 μg/ml; 11, berberine 0.5 μg/ml; 12, berberine 5.0 μg/ml. The result is the representative of three separate experiments and the results were reproducible.

Composition analysis of RIC compare with berberine

RIC was composed of 30% of crude protein, 20% of reducing sugar, and 10% of crude ash. We further analyzed the component of RIC with HPLC. RIC contains alkaloids as their ingredients. As shown in Fig. 6, RIC contained berberine which is approximately 10 % in total ingredients.

Discussion

We report here that RIC, one of natural products, has anti-HIV activity. RIC is mixed extracts from two herbs traditionally used for oriental medicine. The organic solvent extract of *Ricinus communis*, Oleum Ricini, is 'caster oil' which is used as a stimulant laxatives (Brunton, 1991). Oleum Ricini have ricin and ricinoleic acid as major components which have toxicity. But these toxicants were successfully removed during extraction processes because of their high lipid solubilities. The major components of Coptidis Rhizoma are berberine which is classified to isoquinoline alkaloids, and Coptidis contains palmatine, jateorrhizine, coptisine, and magnoflorine. Because materials for oriental medicine have been used several hundreds years, toxicity of these materials cannot be severe obstacle in

the development of drug for the treatment of patients.

From the composition analysis of RIC, Ricini, and Coptidis with HPLC, new compounds which were not present in Ricini or Coptidis extract were detected in combinational extract, RIC (unpublished data). We can test possible antiviral activity of newly generated components by using combinational extracts. Many herbs are combined in oriental medicine for their activity. It is possible that new therapeutic compounds may be generated from combined herbs.

RIC markedly inhibited syncytia formation (Table I), reverse transcriptase activity (Fig. 3), and CAT activity (Fig. 5). So we suggest that RIC could be developed as a good anti-HIV drug. In a comparison with AZT, RIC showed more effective inhibition of reverse transcriptase activity at a concentration which induced complete inhibition of syncitium formation. However, RIC showed marginal cytotoxicity in the SupT1 cells with 10 $\mu g/ml$ (Table I). The extracts of these herbs are being used as oriental medicines and there may be tolerant mechanisms against RIC in human body. The LD_{50} values of RIC were 136.31 mg/kg B.W. (male) and 140.76 mg/kg B.W. (female) s.c., respectively (unpublished data). Adult contains approximately 3.5 liters of plasma. If RIC can be injected 35 mg intravenously to make 10 $\mu g/ml$ concentration without severe toxic effect it may be useful to treat AIDS patients.

RIC did not block reverse transcriptase activity directly (Fig. 4) whereas RIC still inhibit reverse transcriptase activity in pSVCAT-infected H9 cells (Fig. 3). These data suggest that RIC is uptaken into cells and inhibits one of critical steps in the replication of HIV rather than it inhibits the step of reverse transcriptase reaction in the life cycle of HIV.

For the IC_{50} values RIC has approximately 2.5 $\mu g/ml$ and one of major components, berberine has 0.5 $\mu g/ml$ in the inhibition of syncytia formation. However, 2 $\mu g/ml$ of RIC and 0.5 $\mu g/ml$ of berberine have similar effect in the inhibition of reverse transcriptase and chloramphenicol acetyltransferase. The extract of RIC contains berberine approximately 10% (Fig. 6). Therefore there is a possibiltiy that another component besides berberine may also act to inhibit viral replication. It might be necessary to fractionate RIC extract to identify the structures of active components besides berberine. It is also possible that the purified active components themselves are toxic in human body. Another unknown components may be needed to decrease the toxicity. It will be valuable the characterization of each component involved in the antiviral action of RIC for further study.

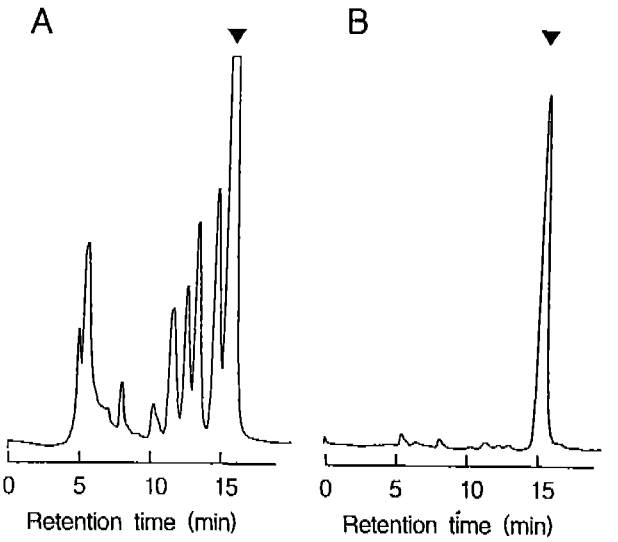

Fig. 6. HPLC chromatograms of RIC and berberine. The assay prodedure was described in Materials and Methods. Arrowheads in chromatograms show the peak of berberine. A, RIC (500 $\mu g/ml$) 20 μl was injected for the analysis. B, standard berberine (43.75 $\mu g/ml$) 10 μl was injected.

Acknowledgement

We thank Mr. A. H. Lee and M. K. Song and Ms. E. K. Suh for the excellent technical assistance. This work was supported by grant from the Korea Association of Creation Research.

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