J. Food Sci. Nutr.
Vol. 5, No. 3, p. 170~173 (2000)

Inhibitory Effects of Various Edible Plants and Flavonoids from the Leaves of *Cedrela sinensis* on Human Immunodeficiency Virus Type 1 Protease

Jong Cheol Park†, Jong Moon Hur, Ju Gwon Park, Hyun Joo Kim*, Kyeong Hee Kang*, Myeong Rak Choi and Sang Ho Song****

Department of Oriental Medicine Resources, Sunchon National University, Sunchon 540-742, Korea
**Department of Cosmetology, Sunchon Chongam College, Sunchon 540-743, Korea*
***Department of Biotechnology, Yosu National University, Yosu 550-749, Korea*

Abstract

The inhibitory effect of extracts from 15 edible plants on the protease of human immunodeficiency virus (HIV) type 1 was investigated. Protease activity was determined by incubating the extracts in a reaction mixture containing protease and substrate His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2 to inhibit proteolytic cleavage. Of various plants tested, the leaves of *Cedrela sinensis* inhibited the HIV-1 protease by 42% at a concentration of 100 μg/ml. A major flavonoid isolated from the leaves of *C. sinensis*, quercetin 3-O-α-L-rhamnoside showed inhibitory activity of 19% at a concentration of 100 μM.

Key words: HIV-1 protease, protease inhibitor, edible plant, *Cedrela sinensis*, flavonoid

INTRODUCTION

The etiological agent of AIDS has been identified as human immunodeficiency virus (HIV), and two genetically distinct subtypes, HIV-1 and HIV-2, have been characterized (1). Much research associated with anti-AIDS drugs is currently aimed at developing novel agents to inhibit the replication of HIV-1 through various targets. Various extracts from traditional medicine in India, China, Egypt, Indonesia and Panama were screened *in vitro* for activity which inhibits the formation of HIV and giant cells in HIV infected cells (2-7). There are many possible ways to block the normal multiplication of HIV-1. One of them is to block the normal action of HIV-1 protease so that HIV-1 protease can not effectively produce regulatory, structural and maturation proteins from their precursor, polyprotein. HIV-1 protease has been demonstrated to play an essential role in viral replication (8). It is considered as a potential target for anti-AIDS therapy.

For the purpose of finding specific inhibitors of HIV-1 protease, we examined various edible plants for the inhibitory activity of protease, by determining the proteolytic activity of protease using HPLC.

MATERIALS AND METHODS

Plant materials

Edible plants were collected in Sunchon on April 1995. Five grams of each edible plant was extracted with methanol under reflux for 3 h. The solvent was removed under reduced pressure to give dry extracts.

Isolation and identification of flavonoids from *Cedrela sinensis*

The powdered leaves of *Cedrela sinensis* A. Juss (voucher species No. NM016; 700 g) was refluxed with MeOH. The MeOH extract (115 g) was partitioned with CH_2Cl_2 (36 g), *n*-BuOH (22 g) and H_2O (48 g) fractions, respectively. The *n*-BuOH fraction (15 g) was subjected to silica gel chromatography with $CHCl_3$-MeOH-H_2O (7:3:1, lower layer) and $CHCl_3$-MeOH-H_2O (65:35:10, lower layer) as eluants to give B-1 ~ B-15 subfractions (volume of each tube: 30 ml). We isolated pure compound 1 (1,250 mg) from subfr. B-6 (tubes no. 45 ~77), compound 2 (33 mg) from subfr. B-8 (tubes no. 98 ~ 105), compound 3 (98 mg) from B-13 (tubes no. 183 ~ 196), respectively.

The NMR spectra were recorded with a Brucher AM-200 spectrometer containing TMS as an internal standard and chemical shifts were given as δ (ppm).

Compound 1 (quercetin 3-O-α-L-rhamnoside)
^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 12.6 (1H.s, 5-OH), 7.29 (1H, d, J=2.0Hz, H-2′), 7.24 (1H, dd, J=2.0Hz and 8.2Hz, H-6′), 6.65 (1H, d, J=8.2Hz, H-5′), 6.38 (1H, d, J=1.9Hz, H-8), 6.20 (1H, d, J=1.9Hz, H-6), 5.24 (1H, d, J=1.3Hz, anomeric H), 0.80 (3H, d, J=5.5Hz, -CH_3), ^{13}C-NMR (DMSO-d_6, 50.3 MHz) δ : 177.8 (C-4), 164.2 (C-7), 161.3 (C-5), 157.3 (C-2), 156.4 (C-9), 148.5 (C-4′), 145.2 (C-3′), 134.2 (C-3), 121.1 (C-1′), 120.7 (C-6′), 115.7 (C-5′), 115.5 (C-2′), 104.1 (C-10), 101.6 (C-1″), 98.7 (C-6), 93.6 (C-8), 71.2 (C-4″), 70.6 (C-5″), 70.4 (C-3″), 70.1 (C-2″), 17.5 (C-6″)

Compound 2 (quercetin 3-O-β-D-glucoside)
^{1}H-NMR (DMSO-d_6, 200 MHz) δ : 7.59 (1H, d, J=2.0 Hz,

†Corresponding author. E-mail. jcpark@sunchon.ac.kr
Phone. 82-61-750-3662, Fax: 82-61-752-8551

H-2′), 7.56 (1H, dd, J=9.0&2.0 Hz, H-6′), 6.83 (1H, d, J=9.0 Hz, H-5′), 6.39 (1H, d, J=1.8Hz, H-6), 6.19 (1H, d, J=1.8 Hz, H-8), 5.46 (1H, d, J=7.0 Hz, anomeric H); ^{13}C-NMR (DMSO-d_6, 50.3 MHz) δ : 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-2), 156.2 (C-9), 148.4 (C-4′), 144.8 (C-3′), 133.3 (C-4), 121.6 (C-6′), 121.1 (C-1′), 116.2 (C-5′), 115.2 (C-2′), 103.9 (C-10), 100.6 (C-1″), 98.6 (C-6), 93.5 (C-8), 77.6 (C-5″), 76.5 (C-3″), 74.1 (C-2″), 69.9 (C-4″), 60.9 (C-6″)

Compound 3 (quercetin 3-O-rutinose)

^{1}H-NMR (DMSO-d_6 200 MHz) δ : 12.58 (1H, s, C_5-OH), 7.56 (1H, dd, J=9.0 & 1.9 Hz, H-6′), 7.51 (1H, d, J=1.9 Hz, H-2′), 6.83 (1H, d, J=9.0 Hz, H-5′), 6.38 (1H, d, J=1.8 Hz, H-8), 6.17 (1H, d, J=1.8 Hz, H-6), 5.32 (1H, d, J=7.2 Hz, anomeric H of glucose), 4.37 (1H, s, anomeric H of rhamnose), 0.98 (3H, d, J=6.0 Hz, CH_3 of rhamnose); ^{13}C-NMR (DMSO-d_6, 50.3 MHz) δ : 177.3 (C-4), 164.1 (C-7), 161.2 (C-5), 156.6 (C-2), 156.4 (C-9), 148.4 (C-4′), 144.7, (C-3′), 133.3 (C-3), 121.6 (C-1′), 116.3 (C-5′), 115.2 (C-2′), 104.0 (C-10), 101.2 (C-1″), 100.7 (C-1‴), 98.7 (C-6), 93.6 (C-8), 76.4 (C-3″), 75.9 (C-5″), 74.1 (C-2″), 71.9 (C-4‴) 70.6 (C-4″), 70.4 (C-2‴), 70.0 (C-3‴), 68.2 (C-5‴), 67.0 (C-6″), 17.7 (C-6‴)

Assay for inhibition of HIV-protease

HIV-1 protease, a generous gift from Prof. M. Hattori, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan, was prepared as described previously (6). The enzyme was dissolved in a buffer solution composed of 50 mM NaOAc (pH 5.0, 1 mM EDTA, 2 mM 2-mercapto ethanol and 25% glycerol). A peptide having an amino acid sequence corresponding to the p24-p15 cleavage site, His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2 was obtained from peptide institute, Inc. (Osaka, Japan), and dissolved in 50 mM NaOAc (pH 5.0) to give a concentration of 2 mg/ml. The extracts and components tested were dissolved in dimethyl sulfoxide (10% in the reaction mixture). The sample solutions were made at a concentration of 500 μg/ml. In the reaction mixture, the sample solution (1 μl) was diluted to a total volume of 5 μl, giving a concentration of 100 μg/ml. A reaction mixture (5 μl) composed of 1 μl of 50 mM NaOAc (pH 5.0), 1 μl of a substrate solution, 1 μl of a test sample and 2 μl of an HIV-1 protease solution was stirred, centrifuged and then incubated at 37°C for 1 h. The reaction was terminated by heating at 90°C for 1 min. The volume was then adjusted to 40 μl with distilled water. A control reaction was performed under the same conditions by using the solvent instead of the sample in the reaction mixture.

HPLC

An LC9A liquid chromatograph and an SPC-6A UV spectrophotometric detector (Shimazu Co., Kyoto) were used. Five μl of the reaction mixture was injected by an auto-injector (Shimazu SIL-6B) into a column (150×4.6 mm, ODS-AP-302, YMC), which was eluted with a linear gradient of acetonitrile (20～40%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The (*p*-NO_2-Phe)-bearing hydrolysate and substrate were eluted at 3.91 and 9.82 min, respectively. The activity of HIV-1 protease was calculated from the ratio of the product peak-area to the substrate peak-area by using an integrator C-R6A Chromatopac (Shimadzu, Japan). The inhibition (%) was calculated as follows; Inhibition (%)=(CP-SP)/CP×100, where CP is the control product ratio and SP is the sample's product ratio.

RESULTS AND DISCUSSION

Many researchers independently discovered the causative agent of AIDS at approximately the same time (1983-4) and named the virus responsible: LAV, the lymphadenopathy-associated virus; HTLV-III, the human T-cell leukaemia (lymphotropic) virus, type III; and ARV, the AIDS-associated retrovirus. In 1986, a subcommittee of the International Committee on the Taxonomy of Viruses proposed that the AIDS retroviruses should be officially designated as human immunodeficiency viruses. This has become the standard term for the viruses which can cause immunosuppression in humans and refers to two viruses: HIV-1, the predominant AIDS-causing virus in the world, and HIV-2, a biologically distinct second type of AIDS virus, identified in 1986 and generally restricted to West Africa (9).

HIV possesses some enzymes for viral replication, such as RNA-dependent DNA polymerase or reverse transcriptase, ribonuclease H, integrase and protease. HIV-protease is considered to be a good target for the development of anti-HIV drugs, since it plays an important role in the process of maturation and infection of the virus. This protease functions as a protein dimer of 11 kDa each, which possess a residue of Asp-Thr-Gly as the catalytic site, and the amino acid sequence subjected to cleavage by protease includes Phe-Pro, Pro-Tyr and Leu-Phe in polyprotein (10).

We investigated the inhibitory effect of methanol extracts from 15 edible plants on HIV-1 protease. From these samples, the extract of the leaves of *C. sinensis* (No. 7) showed a moderate inhibitory effect (>40%) at a concentration of 0.1 μg/ml against HIV-1 protease (Table 1). Other extracts, such as those of the aerial part of *Cirsium japonicum* var. *ussuriense* (No. 9), the whole plant of *Allium monanthum* (No. 1), leaves of *Perilla frutescens* var. *japonica* (No. 13) and *Eriobotrya japonica* (No. 10), aerial parts of *Angelica keiskei* (No. 3), *Oenanthe javanica* (No. 12) and *Artemisia princeps* var. *orientalis* (No. 4), and the whole plant of *Capsella bursa-pastoris* (No. 5) showed weak inhibitory activities (10～30%).

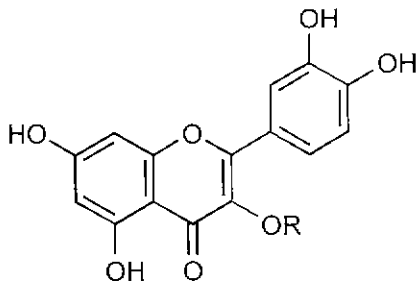
We studied components of the extract from leaves of *C. sinensis* A. Juss (Meliaceae), which showed activity which inhibits HIV protease. The leaves and stem of this plant have been used for the treatment of enteritis, dysentery and itch in oriental medicine (11). The leaves of *C. sinenesis* were extracted with methanol and the extract was partitioned with

Table 1. Inhibitory effects of edible plants on HIV-1 protease

No.	Scientific name	Korean name	Family	Part used	Inhibition (%)
1	*Allium monanthum* Max.	Dal-rae	Liliaceae	whole plant	18.8±0.2[1)]
2	*Allium tuberosum* Roth.	Bu-chu	Liliaceae	aerial part	-5.9±0.3
3	*Angelica keiskei* Koidz.	Sin-seon-cho	Umbelliferae	aerial part	22.8±1.8
4	*Artemisia princeps* var. *orientalis* (Pampan.) Hara	Tsug	Compositae	aerial part	22.8±0.9
5	*Capsella bursa-pastoris* (L.) Medicus	Naeng-i	Cruciferae	whole plant	22.1±1.8
6	*Capsicum annuum* L.	Go-chu	Solanaceae	leaves	11.5±0.7
7	*Cedrela sinensis* A. Juss.	Cham-jug	Meliaceae	leaves	41.7±0.8
8	*Chrysanthemum coronarium* var. *spatiosum* Bailey	Tsug-gag	Compositae	aerial part	7.3±1.5
9	*Cirsium japonicum* var. *ussuriense* Kitamura	Eong-geong-ki	Compositae	aerial part	26.0±0.9
10	*Eriobotrya japonica* Lindl.	Bi-pa	Rosaceae	leaves	14.6±0.8
11	*Glycine max.* Merr.	Kong	Leguminosae	leaves	2.8±0.8
12	*Oenanthe javanica* (BL.) DC.	Mi-na-ri	Umbelliferae	aerial part	12.9±4.1
13	*Perilla frutescens* var. *japonica* Hara	Deul-kae	Labiatae	leaves	15.7±0.6
14	Sedum sarmentosum Bunge	Dol-na-mul	Crassulaceae	whole plant	7.0±5.0
15	*Youngia sonchifolia* Max.	Go-deul-pae-gi	Compositae	whole plant	-13.8±1.9

[1)]The results are the mean±S.E. of 3 replications.

CH_2Cl_2, *n*-BuOH and H_2O. The *n*-BuOH fraction was subjected to silica gel chromatography to give compounds 1, 2 and 3, which were elucidated as well-known flavonoids, quercetin 3-O-α-L-rhamnoside, quercetin 3-O-β-D-glucoside and quercetin 3-O-rutinoside, respectively by comparison of reported NMR data (12). The detailed assignments of flavonoids and their chemical structures are shown in materials and methods, and Fig. 1, respectively. A major flavonoid, quercetin 3-O-α-L-rhamnoside inhibited the activity of HIV-1 protease by 19% at a concentration of 100 μM. The inhibitory effects at 100 μM were 12% by quercetin 3-O-β-D-glucoside and 7% by quercetin 3-O-rutinoside, respectively (Table 2). However, more active substances seemed to be present in the extract of this plant. Further studies on the isolation of potent anti-HIV-protease components from *C. sinensis* are now in progress.



R	
1	rhamnose
2	glucose
3	rutinose

Fig. 1. Flavonoid structures from *Cedrela sinensis*.
1: quercetin 3-O-α-L-rhamnoside
2: quercetin 3-O-β-D-glucoside
3: quercetin 3-O-rutinoside

Table 2. Inhibitory effect of flavonoid glycosides isolated from leaves of *Cedrela sinensis* on the HIV-1 protease

Compound	Inhibition (%)
Quercetin 3-O-α-L-rhamnoside	19.4±5.1[1)]
Quercetin 3-O-β-D-glucoside	11.6±1.1
Quercetin 3-O-rutinoside	7.2±1.4

[1)]The results are the mean±S.E. of 3 replications.

ACKNOWLEDGEMENTS

We thank Prof. M. Hattori and Assistant Prof. H. Miyashiro, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan, for the HIV-protease assay.

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(Received June 26, 2000)