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Inhibitory Effects of Methanol Extracts from Korean Medicinal Plants against HIV-1 Protease Activity

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ABSTRACT : Korean medicinal plants were screened for their inhibitory activity against HIV-1 protease. The inhibitory activity of protease was determined by incubating the extracts in reaction mixtures containing protease and substrate His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2 to perform proteolytic cleavage reactions. In this study the twenty six extracts from medicinal plants were investigated. Of the extracts tested, the extracts from the stem of *Morus alba* exhibited the strongest activity with inhibition of 81% at a concentration of 100 ㎍/㎖. The extracts of the flower of *Saxifraga stolonifera*, and stems of *Euonymus japonica* and *Castanea crenata* showed appreciable inhibitory activity (>50%) against HIV-1 protease at same concentration.

Key words : HIV-1 protease, protease inhibitor, *Morus alba*, Korean plant

INTRODUCTION

AIDS is still a threatening disease world-wide. HIV-1 is the causative agent of AIDS and is a member of retrovirus group. The development of antiretroviral therapy against AIDS has been an intense research effort since the discovery of the causative agent. HIV possesses some enzymes that work on viral replication, such as reverse transcriptase, integrase and protease. Genetic and biochemical studies have demonstrated that protease activity is essential for the proper assembly and maturation of fully infectious virions of HIV (Kohl *et al.*, 1988). Therefore, HIV-1 protease has become an important target for the design of antiviral agents for AIDS.

In the course of our continuing search for plants as anti-HIV agent sources, we have reported the inhibitory effects of Korean medicinal plants (Hur *et al.*, 2002; Park *et al.*, 2000a; Park *et al.*, 2000b; Park *et al.*, 2000c; Park *et al.*, 2002; Yu *et al.*, 1998) on HIV-1 protease. In this paper, we describe the investigation of 26 extracts from Korean medicinal plants for their inhibitory effects against HIV-1 protease.

MATERIALS AND METHODS

Preparation of the extracts

The plant resources were collected by one (J.C.P.) of the authors. Voucher specimens are deposited in Herbarium of Sunchon National University, Korea. Five grams of each plant were refluxed separately with methanol for 3 hours. The extracts were concentrated and freeze-dried.

HIV-1 protease assay

The fused recombinant HIV-1 protease for the

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screening of plant extracts was prepared in our laboratory as reported previously (Kusumoto *et al.*, 1995). A substrate, His-Lys-Ala-Arg-Val-Leu-(pNO$_2$-Phe)-Glu-Ala-Nle-Ser-NH$_2$, was purchased from Peptide Institute, Inc. (Osaka, Japan). The extracts were dissolved in dimethyl sulfoxide (10% in the reaction mixture). 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 the plant extract solution, and 2 μℓ of HIV-1 protease solution, was stirred, centrifuged and then incubated at 37℃ for one hour in a microtube. A control reaction was done under the same condition without plant extract. The reaction was stopped by heating microtubes at 90℃ for one minute. 35 μℓ of autoclaved water was added into the reaction mixture and an aliquot of 5 μℓ was analyzed by HPLC. The hydrolysate and the remained substrate were quantitatively analyzed by HPLC under the following conditions: Column, RP-C18 (150×4.6 ㎜ i.d., YMC Co., Kyoto, Japan); elution, a linear gradient of CH$_3$CN (20~40%) in 0.1% TFA; injection volume, 5 μℓ; flow rate, 1.0 μℓ/min.; detection, 280 ㎚; system controller, Shimadzu SCL-6B; pump, Shimadzu LC-9A; detector, Shimadzu SPD-6A; recorder & integrator, Shimadzu C-R6A chromatopac; autoinjector, Shimadzu SIL-6B (all Shimadzu Co., Kyoto, Japan). The retention times of the substrate and Phe(NO$_2$)-bearing hydrolysate were approx. 9 and 4 minutes, respectively. The inhibitory activity in the HIV-1 protease reaction was calculated from the ratio of the substrate peak area to the product peak area.

RESULTS AND DISCUSSION

Zidovudine has been reported to decrease both the mortality and frequency and severity of opportunistic infections in a select group of patients infected with HIV (Fischl *et al.*, 1987). However, the administration of Zidovudine has also been associated with substantial toxicity, primarily hematologic in nature (Richman *et al.*, 1987; Schmitt *et al.*, 1988; Larder *et al.*, 1989). Therefore, the development of specific and potent anti-AIDS drugs to restrain infection by HIV remains an urgent need.

In HIV-1, the *gag* and *gag-pol* genes are translated as two polyproteins, which are subsequently cleaved by the action of a virus-encoded protease into the four structural gag proteins of the virion core, for example, p17, p9, p7 and p24, together with the *pol*-encoded enzymes essential for retrovirus replication, such as the protease itself, reverse transcriptase and intergrase (Robert, 1988). The HIV life cycle consists of more than a dozen steps; interrupting any one of them can prevent the virus from reproducing itself. In the first step of replication, reverse transcriptase transcribes the viral RNA into a double strand DNA. Then, this DNA is integrated into the host chromosome, and the viral components are synthesized and assembled into new virus. The maturation of the virus takes place at the last step by viral protease, which cleaves the viral polyproteins at the specific amino acid sequences to give functional proteins or enzymes. The mature viruses bud from the cells and continuously infect other T-cells. HIV protease being a member of the aspartic protease family is regarded as one of the most promising targets for development of anti-HIV agent (Kohl *et al.*, 1988).

For the purpose of finding the natural resources having anti-HIV protease activity, we examined the medicinal plants by determining the proteolytic activity of protease by HPLC. Among the twenty six methanol extracts from medicinal plants, the stem of *Morus alba* (No. 11) showed the most potent inhibitory effect against HIV-1 protease by 81% at a concentraion of 100 μg/mℓ. The root bark of *Morus alba* has been used as a blood pressure depressant, an expectorant, a diuretic and a laxative in traditional Chinese medicine. The flavones and stilbene constituents were isolated as active principles from the root bark (Qiu *et al.*, 1996). The leaves of this plant have also been used as a blood pressure depressant, and scopolin, skimmin and roseoside II were isolated from same parts (Doi *et al.*, 2001). The remarkable inhibitory effects (>50%) were observed in methanol extracts of the stem of *Castanea crenata* (No. 1), the flower of *Saxifraga stolonifera* (No. 19) and the stem of *Euonymus japonica* (No. 6) and at same concentration. The root of *Humulus japonicus* (No. 7) and the leaves of *Smilax china* (No.21)

Table 1. Inhibitory effect of the methanol extracts from Korean medicinal plants on HIV-1 protease activity.

No.	Scientific name	Family name	Part used	Inhibition (%)†
1	*Castanea crenata* S. et Z.	Fagaceae	Stem	61.81± 3.3
2	*Chelidonium majus* var. *asiaticum* (Hara) Ohwi	Papaveraceae	Flower	-5.10±19.1
3	*Chenopodium album* var. *centrorubrum* Makino	Chenopodiaceae	Stem	15.43±11.3
4	*Chenopodium album* var. *centrorubrum* Makino	Chenopodiaceae	Root	29.16± 4.6
5	*Chenopodium virgatum* Thunb.	Chenopodiaceae	Aerial part	17.39± 8.2
6	*Euonymus japonica* Thunb.	Celastraceae	Stem	53.48± 3.0
7	*Humulus japonicus* S. et Z.	Cannabinaceae	Root	45.18± 4.7
8	*Lactuca indica* var. *laciniata* (O. Kuntze) Hara	Compositae	Root	-29.20±36.2
9	*Lonicera japonica* Thunb.	Caprifoliaceae	Stem	-6.56± 5.3
10	*Lygodium japonicum* (Thunb.) Sw.	Schizaeaceae	Root	-25.37±19.1
11	*Morus alba* L.	Moraceae	Stem	81.34± 1.9
12	*Photinia glabra* (Thunb.) Max.	Rosaceae	Leaf	-23.31± 4.7
13	*Phytolacca esculenta* V. Houtte	Phytolaccaceae	Stem	12.50± 2.3
14	*Phytolacca esculenta* V. Houtte	Phytolaccaceae	Leaf	19.96±12.8
15	*Phytolacca esculenta* V. Houtte	Phytolaccaceae	Fruit	24.85± 2.1
16	*Poncirus trifoliata* Rafin.	Rutaceae	Stem	27.25±12.5
17	*Portulaca oleracea* L.	Portulacaceae	Root	17.46± 9.1
18	*Pulsatilla koreana* Nakai	Ranunculaceae	Aerial part	24.84± 3.9
19	*Saxifraga stolonifera* Meerb.	Saxifragaceae	Flower	54.90± 5.8
20	*Saxifraga stolonifera* Meerb.	Saxifragaceae	Root	8.01± 6.3
21	*Smilax china* L.	Liliaceae	Leaf	42.80± 5.2
22	*Taraxacum coreanum* Nakai	Compositae	Aerial part	14.80± 5.3
23	*Taraxacum coreanum* Nakai	Compositae	Root	18.04±12.2
24	*Thuja orientalis* L.	Cupressaceae	Stem	28.39± 4.4
25	*Thuja orientalis* L.	Cupressaceae	Fruit	28.87± 0.8
26	*Viola mandshurica* W. Becker	Violaceae	Root	2.63± 9.2

†The concentration of the extract was 100 μg/mℓ and results are the mean±SD (n=3).

showed the inhibitory effects on HIV-1 protease by 45 and 43%, respectively. Other, such as the root of *Chenopodium album* var. *centrorubrum* (No. 4), the fruit (No. 25) and stem (No. 24) of *Thuja orientalis*, the aerial part of *Pulsatilla koreana* (No. 18), the stem of *Poncirus trifoliata* (No. 16) and the fruit of *Phytolacca esculenta* (No. 15) showed weak inhibitory activities (25~29%). Further study on anti-HIV-protease components from *Morus alba* is now in progress.

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