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Inhibitory Effects of Ninety Nine Korean Plants on Human Immunodeficiency Virus Type 1 Protease Activity

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Abstract

Ninety nine extracts from Korean plants were screened for their inhibitory activities on human immunodeficiency virus (HIV) type 1 protease by an HPLC method. The protease inhibitory activities were 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. Of the extracts tested, the water extracts of *Viburnum awabuki* (stem and leaves) and *Distylium racemosum* (leaves) had the highest protease inhibitory activities at a concentration of 100 μg/mL. Activity-guided fractionation, revealed that the *n*-butanol fraction of the *V. awabuki* extract and the ethyl acetate fraction from the *D. racemosum* extract had the greatest inhibitory activity on HIV-1 protease.

Key words: HIV-1 protease, protease inhibitor, Korean plants

INTRODUCTION

Various researchers independently discovered the causative agent of acquired immunodeficiency syndrome (AIDS) at approximately the same time (1983~4). In May 1986, a subcommittee of the International Committee on the Taxonomy of Viruses proposed that the AIDS retroviruses be officially designated as the human immunodeficiency viruses (HIV). This has become the standard term for the viruse that causes immunosuppression in humans (1).

During the course of our continuing search for plants as anti-AIDS agents, we have reported the inhibitory effects of various medicinal plants from India, China, Egypt, Indonesia and Panama on HIV and giant cell formation in HIV infected cells (2-6).

The HIV life cycle consists of more than a dozen steps; interrupting any one of them could prevent the virus from reproducing itself. Recently, characterization of the structure and function of a protease has shown another target in HIV (7). 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. The four structural proteins, designated p17, p9, p7 and p24, together with pol-encoded enzymes, including the protease itself, reverse transcriptase, ribonuclease and endonuclease, are essential for retroviral replication (8).

Since the cleavage initiates the process of maturation of the virion, and makes the virus infections (9), inhibition of the HIV-protease is considered to be a promising target for anti-HIV drugs.

In this paper we report the results of our screening of 99 native Korean plants for inhibition of the HIV-1 protease enzyme.

MATERIALS AND METHODS

Preparation of the extracts

All plants were collected and authenticated by one (JCP) of the authors. Voucher specimens are deposited in the Herbarium at Sunchon National University, Korea. Five grams of each plant was refluxed separately with methanol or water for 3 hours, and then concentrated and freeze-dried.

Assay for inhibition of HIV-protease

Fused recombinant HIV-1 protease was prepared in our laboratory as reported previously (2). The enzyme was dissolved in a 50 mM NaOAc (pH 5.0) buffer solution (50 mM NaOAc, 1 mM EDTA, 2 mM 2-mercaptoethanol 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 ob-

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tained from Peptide Institute, Inc. (Osaka, Japan), and dissolved in the 50 mM NaOAc buffer solution (pH 5.0) to a concentration of 2 mg/mL. The extracts tested were dissolved in distilled water or 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. Reaction mixtures (5 μL total volume) composed of 1 μL of the 50 mM NaOAc (pH 5.0), 1 μL of the substrate solution, 1 μL of a sample solution and 2 μL of the HIV-1 protease solution were stirred, centrifuged and then incubated at 37°C for 1 hour in a microtube. 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, but using the solvent instead of the sample in the reaction mixture.

HPLC

The product and the substrate were quantitatively analyzed by HPLC under the following conditions: column, RP-C18 (150×4.6 mm i.d., YMC Co., Kyoto, Japan); elution, a linear gradient of CH_3CN (20~40%) in 0.1% TFA; injection volume, 5 μL; flow rate, 1.0 mL/min; detection, 280 nm; system controller, Shimadzu SCL-6B; pump, Shimadzu LC-9A; detector, Shimadzu SPD-6A; recorder & integrator, Shimadzu C-R 6A chromatopac; autoinjector, Shimadzu SIL-6B (all Shimadzu Co., Kyoto, Japan). Following HPLC separation, the product and substate were detected by a UV spectrophotometric detector. The retention times were approximately 4 min. for the product and 9 min. for the substrate, respectively. The rate of inhibition could be calculated as follows: Activity=Area product / ($Area_{substrate}$+$Area_{product}$); Inhibition (%) = ($Activity_{control}$ − $Activity_{sample}$)/$Activity_{control}$ × 100.

RESULTS AND DISCUSSION

AIDS has become a global pandemic, posing a serious public health threat to most of the developed and developing world. HIV-1, the causative agent of AIDS, is a retrovirus. Many scientists are currently investigating numerous approaches aimed at developing novel agents to arrest the replication of HIV through various targets. HIV possesses several enzymes that are essential for viral replication, such as RNA-dependent DNA polymerase or reverse transcriptase, integrase and protease. In the first step of replication, reverse transcriptase transcribes the viral RNA into a double strand DNA. The DNA is then 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 using viral protease, which cleaves the viral polyproteins at specific amino acid sequences to give functional proteins or enzymes. The mature viruses bud from the cells and continuously infect other T-cells.

HIV-1 protease has been demonstrated to play an essential role in the viral replication (10). It is considered to be a potential target for anti-AIDS therapy, since the inhibition of this enzyme produces immature and non-infectious virions (11,12). The HIV-1 protease has a molecular weight 11,000 daltons, and is an aspartic protease containing a Asp-Thr-Gly residue as its active site.

A range of HIV-1 protease inhibitors has been designed and applied in clinical trials such as Saquinavir, Rionavir and Indinavir. However, the development of drug resistance by the virus, irrespective of the target, remains as an overwhelming problem in AIDS-chemotherapy (13). Thus, there is a great need to develop and search for new and different anti-HIV candidates.

As part of our work on naturally occuring antiviral agents, we screened a wide variety of Korean plants for inhibition of HIV-1 protease. The inhibitory activities of 104 methanol and water extracts of the plants against the HIV-1 protease are presented in Table 1. At a concentration of 100 μg/mL, the most potent inhibitory effect was observed in the water extract of the stem of *Viburnum awabuki* (No. 94) with 91.7% inhibition. The water extracts of the leaves of *Distylium racemosum* (No. 24) and *Viburnum awabuki* (No. 93) were also potent inhibitors, with inhibitions of 70.3 and 69.3%, respectively.

Three other extracts with appreciable inhibitory activity (>50%) against HIV-1 protease were; the water extract of the stem of *Distylium racemosum* (No. 25), the methanol extracts of the root of *Physalis alkekengi* var. *francheti* (No. 67) and the stem of *Platycarya strobilacea* (No. 70). The methanol extracts of aerial parts of *Alisma plantago-aquatica* var. *orientale* (No. 7) and *Lactuca indica* var. *laciniata* (No. 44), the leaves of *Euonymus alatus* (No. 30), *Morus alba* (No. 56), *Quercus myrsinaefolia* (No. 73), *Sageretia theezans* (No. 77) and *Taxodium distichum* (No. 89) and the stem of *Smilax china* (No. 83) and the root of *Suaeda asparagoides* (No. 88), also showed moderate inhibitory activities (>30%).

To fractionate active components, the extracts from the stem and leaves of *V. awabuki* and leaves of *D. racemosum* were serially extracted with dichloromethane, ethyl acetate and *n*-butanol. The inhibitory effects of each fraction from the extracts are shown in Table 2. The inhibitory effects of *n*-butanol fractions from the stem and leaves of *V. awabuki* were stronger than the others. The dichloromethane, ethyl acetate, and *n*-butanol fractions of *D. racemosum* inhibited the protease by 51.1%,

Table 1. Inhibitory effects of Korean plant extracts on HIV-1 protease

No.	Scientific name	Family name	Part used	Extract	Inhibition (%)
1	*Acanthopanax koreanum* Nakai	Araliaceae	stem	MeOH	15.8± 3.8
2	*Acanthopanax koreanum* Nakai	Araliaceae	flower	MeOH	21.7± 9.2
3	*Achyranthes japonica* (Miq.) Nakai	Amaranthaceae	aerial part	MeOH	6.8± 3.8
4	*Aconitum chiisanense* Nakai	Ranunculaceae	root	MeOH	18.7± 0.1
5	*Aconitum chiisanense* Nakai	Ranunculaceae	aerial part	MeOH	8.1± 0.2
6	*Ajuga decumbens* Thunb.	Labiatae	whole plant	MeOH	17.6± 6.9
7	*Alisma plantago-aquatica* var. *orientale* Samuels.	Alismataceae	aerial part	MeOH	48.5± 1.8
8	*Aralia elata* Seem.	Araliaceae	stem	MeOH	-2.8± 1.4
9	*Aralia elata* Seem.	Araliaceae	rachis	MeOH	3.55± 2.9
10	*Armoracia rusticana* P. Gaertn.	Cruciferae	root	H_2O	7.5± 3.2
11	*Artemisia apiacea* Hance	Compositae	aerial part	MeOH	2.1± 3.7
12	*Castanea crenata* S. et Z.	Fagaceae	leaves	MeOH	14.0± 5.4
13	*Cedrela sinensis* A. Juss.	Meliaceae	petiolule	MeOH	16.6± 4.4
14	*Chelidonium majus* var. *asiaticum* (Hara) Ohwi	Papaveraceae	root	MeOH	22.4± 6.4
15	*Chenopodium album* var. *centrorubrum* Makino	Chenopodiaceae	leaves	MeOH	-3.5± 6.7
16	*Chloranthus glaber* (Thunb.) Makino	Chloranthaceae	stem	MeOH	25.7± 0.9
17	*Chloranthus glaber* (Thunb.) Makino	Chloranthaceae	leaves	H_2O	28.2± 5.2
18	*Colocasia antiquorum* var. *esculenta* Engl.	Araceae	aerial part	MeOH	25.7± 5.2
19	*Commelina communis* L.	Commelinaceae	root	MeOH	-1.8± 0.5
20	*Cudrania tricuspidata* Bureau	Moraceae	stem	MeOH	15.9± 2.8
21	*Cudrania tricuspidata* Bureau	Moraceae	fruit	MeOH	11.2± 5.1
22	*Daphniphyllum macropodum* Miq.	Euphorbiaceae	leaves	MeOH	-37.3± 13.4
23	*Dioscorea batatas* Decne.	Dioscoreaceae	root	MeOH	10.9± 5.2
24	*Distylium racemosum* S. et Z.	Hamamelidaceae	leaves	H_2O	70.3± 7.8
25	*Distylium racemosum* S. et Z.	Hamamelidaceae	stem	H_2O	64.7± 5.4
26	*Elaeagnus umbellata* Thunb.	Elaeagnaceae	leaves	H_2O	23.0± 1.8
27	*Erigeron annuus* (L.) Pers.	Compositae	aerial part	MeOH	3.1± 5.0
28	*Erigeron annuus* (L.) Pers.	Compositae	root	MeOH	40.6± 0.1
29	*Eucommia ulmoides* Oliver	Eucommiaceae	leaves	MeOH	10.4± 0.8
30	*Euonymus alatus* (Thunb.) Sieb.	Celastraceae	leaves	MeOH	35.1± 4.5
31	*Euonymus alatus* (Thunb.) Sieb.	Celastraceae	stem	H_2O	8.7± 5.5
32	*Forsythia koreana* Nakai	Oleaceae	stem	MeOH	29.2± 5.7
33	*Gardenia jasminoides* var. *radicans* Makino	Rubiaceae	stem	H_2O	-1.4± 3.5
34	*Geranium nepalense* subsp. *thunbergii* (S. et Z.) Hara	Geraniaceae	root	MeOH	18.7± 2.6
35	*Gleditsia japonica* var. *koraiensis* (Nak.) Nakai	Leguminosae	leaves	H_2O	7.0± 3.3
36	*Hemistepta lyrata* Bunge	Compositae	root	MeOH	-1.8± 3.4
37	*Hibiscus hamabo* S. et Z.	Malvaceae	root	H_2O	12.6± 6.4
38	*Ilex cornuta* Lindl.	Aquifoliaceae	stem	MeOH	9.4± 3.8
39	*Ilex cornuta* Lindl.	Aquifoliaceae	stem	H_2O	22.2± 2.9
40	*Ilex cornuta* Lindl.	Aquifoliaceae	leaves	MeOH	-8.1± 4.1
41	*Indigofera kirilowii* Max.	Leguminosae	aerial part	MeOH	-8.6± 3.2
42	*Isodon japonicus* (Burm.) Hara	Labiatae	leaves	H_2O	12.6± 4.3
43	*Isodon japonicus* (Burm.) Hara	Labiatae	flower	H_2O	11.1± 6.1
44	*Lactuca indica* var. *laciniata* (O. Kuntze) Hara	Compositae	aerial part	MeOH	38.1± 6.2
45	*Ligustrum lucidum* Ait.	Oleaceae	leaves	H_2O	20.3± 3.1
46	*Ligustrum lucidum* Ait.	Oleaceae	stem	H_2O	-20.3± 0.6
47	*Lindera glauca* Bl.	Lauraceae	leaves	MeOH	2.2± 12.3
48	*Lindera obtusiloba* Bl.	Lauraceae	leaves	MeOH	-27.3± 25.5
49	*Lindera obtusiloba* Bl.	Lauraceae	stem	MeOH	9.2± 3.6
50	*Lindera sericea* (S. et Z.) Bl.	Lauraceae	leaves	MeOH	13.4± 2.7
51	*Litsea japonica* Juss.	Lauraceae	leaves	MeOH	14.2± 4.3
52	*Lonicera japonica* Thunb.	Caprifoliaceae	leaves	MeOH	17.4± 5.2
53	*Machilus japonica* S. et Z.	Lauraceae	leaves	MeOH	-9.3± 12.3
54	*Magnolia grandiflora* L.	Magnoliaceae	stem	MeOH	8.4± 2.9
55	*Magnolia grandiflora* L.	Magnoliaceae	leaves	MeOH	-39.2± 10.1
56	*Morus alba* L.	Moraceae	leaves	MeOH	38.1± 7.1
57	*Morus bombycis* for. *kase* Uyeki	Moraceae	leaves	H_2O	10.6± 2.4
58	*Morus bombycis* for. *kase* Uyeki	Moraceae	stem	H_2O	11.0± 2.0
59	*Morus bombycis* for. *kase* Uyeki	Moraceae	stem	MeOH	23.1± 1.1
60	*Morus bombycis* for. *kase* Uyeki	Moraceae	leaves	MeOH	3.1± 7.1
61	*Nandina domestica* Thunb.	Berberidaceae	stem	MeOH	18.6± 0.5
62	*Nerium indicum* Mill.	Apocynaceae	leaves	MeOH	-26.3± 11.5

Table 1. Continued

No.	Scientific name	Family name	Part used	Extract	Inhibition (%)
63	*Oenothera odorata* Jacq.	Onagraceae	root	MeOH	4.8± 2.9
64	*Parthenocissus tricuspidata* (S. et Z.) Planch.	Vitaceae	stem	MeOH	10.9± 6.5
65	*Phragmites communis* Trin.	Gramineae	root	MeOH	28.7± 9.4
66	*Physalis alkekengi* var. *francheti* (Masters) Hort.	Solanaceae	aerial part	MeOH	14.2± 4.3
67	*Physalis alkekengi* var. *francheti* (Masters) Hort.	Solanaceae	root	MeOH	66.0± 3.7
68	*Phytolacca esculenta* V. Houtte	Phytolaccaceae	root	MeOH	16.4± 4.7
69	*Platycarya strobilacea* S. et Z.	Juglandaceae	stem	H_2O	21.9± 5.6
70	*Platycarya strobilacea* S. et Z.	Juglandaceae	stem	MeOH	57.3± 4.1
71	*Prunella vulgaris* var. *lilacina* Nakai	Labiatae	aerial part	MeOH	1.4± 0.3
72	*Prunella vulgaris* var. *lilacina* Nakai	Labiatae	root	MeOH	25.8± 8.0
73	*Quercus myrsinaefolia* Bl.	Fagaceae	leaves	MeOH	35.9±12.7
74	*Reynoutria elliptica* (Koidz.) Migo	Polygonaceae	stem	MeOH	14.6± 1.1
75	*Reynoutria elliptica* (Koidz.) Migo	Polygonaceae	root	MeOH	-13.3± 0.6
76	*Sageretia theezans* Brongn.	Rhamnaceae	stem	H_2O	27.0± 8.3
77	*Sageretia theezans* Brongn.	Rhamnaceae	leaves	MeOH	34.1± 7.3
78	*Sambucus sieboldiana* Bl.	Caprifoliaceae	stem	H_2O	16.1± 2.0
79	*Sambucus sieboldiana* Bl.	Caprifoliaceae	flower	H_2O	5.7± 7.2
80	*Schisandra nigra* Max.	Magnoliaceae	leaves	H_2O	14.7± 7.1
81	*Schisandra nigra* Max.	Magnoliaceae	stem	H_2O	9.4± 3.2
82	*Serissa japonica* Thunb.	Rubiaceae	leaves	H_2O	13.8± 7.3
83	*Smilax china* L.	Liliaceae	stem	MeOH	32.1± 4.1
84	*Sophora flavescens* Ait.	Leguminosae	flower	MeOH	18.9± 1.1
85	*Sophora flavescens* Ait.	Leguminosae	root	MeOH	19.7± 2.7
86	*Styrax obassia* S. et Z.	Styracaceae	leaves	H_2O	7.6± 3.0
87	*Styrax obassia* S. et Z.	Styracaceae	stem	MeOH	21.2± 8.6
88	*Suaeda asparagoides* (Miq.) Makino	Chenopodiaceae	root	MeOH	38.1± 0.4
89	*Taxodium distichum* (L.) Rich.	Taxodiaceae	leaves	MeOH	48.7± 4.0
90	*Trichosanthes kirilowii* Max.	Cucurbitaceae	aerial part	MeOH	-15.2± 4.2
91	*Ulmus parvifolia* Jacq.	Ulmaceae	leaves	MeOH	6.0± 9.1
92	*Vaccinium bracteatum* Thunb.	Ericaceae	stem	H_2O	26.0± 5.3
93	*Viburnum awabuki* K. Koch	Caprifoliaceae	leaves	H_2O	69.3± 5.4
94	*Viburnum awabuki* K. Koch	Caprifoliaceae	stem	H_2O	91.7± 7.2
95	*Viburnum furcatum* Bl.	Caprifoliaceae	leaves	H_2O	33.8± 7.8
96	*Viburnum furcatum* Bl.	Caprifoliaceae	stem	H_2O	48.4± 0.7
97	*Wistaria floribunda* A.P. DC.	Leguminosae	stem	MeOH	14.5± 1.8
98	*Zanthoxylum piperitum* A.P. DC.	Rutaceae	stem	H_2O	-11.8± 2.9
99	*Zanthoxylum piperitum* A.P. DC.	Rutaceae	root	H_2O	3.5± 0.3

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

Table 2. Inhibitory effects of various fractions against HIV-1 protease

Extract	Fraction	Inhibition (%)
leaves of *Viburnum awabuki*	CH_2Cl_2-soluble	48.1±7.6
	EtOAc-soluble	44.5±0.6
	n-BuOH-soluble	64.6±3.0
stem of *Viburnum awabuki*	CH_2Cl_2-soluble	42.4±9.4
	EtOAc-soluble	38.0±6.7
	n-BuOH-soluble	57.5±0.3
leaves of *Distylium racemosum*	CH_2Cl_2-soluble	51.1±6.6
	EtOAc-soluble	79.2±1.8
	n-BuOH-soluble	18.2±0.6

The concentration of the fraction tested was 100 μg/mL and results are the mean±SD (n=3).

79.2% and 18.2%, respectively. These results suggest that the active constituents of *V. awabuki* and *D. racemosum* might be concentrated in *n*-butanol and ethyl acetate fractions, respectively. *V. awabuki* and *D. racemosum* are evergreen trees which have been widely distributed throughout the Jeju island in Korea. Concerning the chemical constituents present in most active plant extracts, diterpenes, sesquiterpenes and triterpenes have been isolated from *V. awabuki* (14-16). However chemical components on *D. racemosum* have not been studied yet. Further studies on the inhibitory components on HIV-1 protease are now in progress.

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