Phenolic Compounds from *Orostachys japonicus* having Anti-HIV-1 Protease Activity

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Abstract – The water extract of the aerial parts of *Orostachys japonicus* A. Berger showed the inhibitory activity against HIV-1 protease. From the same parts of *O. japonicus*, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, gallic acid and methyl gallate, together with flavonoids, kaempferol, quercetin, kaempferol 3-*O*-β-D-glucoside, kaempferol 3-*O*-β-D-galactoside and quercetin 3-*O*-β-D-glucoside were isolated and characterized by spectral data.

Key words – *Orostachys japonicus* A. Berger, HIV-1 protease, protease inhibition, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, gallic acid, methyl gallate

Introduction

According to the World Health Organization, 5.6 million people were infected with the human immunodeficiency virus (HIV) in 1999 alone, and 33.6 million people are currently living with HIV infection. HIV was identified as the causative agent for Acquired Immunodeficiency Syndrom (AIDS) in 1983 (Barrre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984).

The retroviral protease is absolutely essential for completion of HIV multiplication cycle, and cannot be replaced by any cellular function. Thus protease is an ideal target for the development of anti-AIDS therapy. A large number of HIV-1 protease inhibitors have been developed, and several compounds, namley saquinavir (Bragman, 1996), indinavir, ritonavir (Lea and Faulds, 1996) and nelfinavir (Jarvis and Faulds, 1998) have already been approved for clinical use as anti-AIDS drugs. The major problem regarding the clinical use of protease inhibitors in anti-HIV therapy is the fast emergance of drug-resistant HIV variants (Erickson and Burt, 1996; Klabe *et al.*, 1998). Thus there is a great need to develop and search for new and different anti-HIV candidates.

As a part of our continuing studies to find the anti-

HIV compound from natural resources (Yu *et al.*, 1998), we report in this paper the inhibitory activity of *Orostachys japonicus* A. Berger (Crassulaceae) against HIV-1 protease and isolation of phenolic compounds from title plant. *O. japonicus* is a perenial herbaceous plant which has been traditionally used as an anti-inflammatory agent to treat hepatitis, boils an piles, and also as a hemostatic agent for the treatment of vomiting blood, nose bleeding, and blood excrement (Kim, 1984). And this plant has been used in Korean folk medicine as an anti-cancer agents. Flavonoids and triterpenoids (Park *et al.*, 1991a; 1991b) have been isolated from *O. japonicus*.

Materials and Methods

Plant material – *O. japonicus* was collected in Sunchon, Jonnam, Korea on October 14, 1998. The voucher specimen (NM0359) is deposited in department of Oriental Medicine Resources, Sunchon National University.

Assay for inhibition of HIV-protease – The fused recombinant HIV-1 protease which was a generous gift from Prof. M. Hattori, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Japan, was prepared as reported previously (Kusu- moto *et al.*, 1995). A substrate, His-Lys-Ala-Arg-Val-Leu-(*p*NO$_2$-Phe)-Glu-Ala-Nle-Ser-NH$_2$, was

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purchased from Peptide Institute, Inc. (Osaka, Japan). For the assay of HIV-1 protease, the stock solution of the prepared enzyme was diluted. Five grams of the plant were refluxed with water for 3 hours. The extract was concentrated and dissolved in distilled water. 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 tested extract and 2 μl of an HIV-1 protease solution were stirred, centrifuged and then incubated at 37°C for 1 hr 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, and an aliquot of 5 μl was analyzed by HPLC. A control reaction was performed under the same condition by using the solvent instead of the extract in the reaction mixture.

HPLC – The hydrolysate and the remained 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$_3$CN (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 and integrator, Shimadzu C-R 6A chromatopac; autoinjector, Shimadzu SIL-6B (all Shimadzu Co., Kyoto, Japan). The retention times were 4.6 min. for the product and 10.3 min. for the substrate. The signal is proportional to the concentration of p-NO$_2$-Phe, so the areas of the substrate- and product-peaks reflect the quantity of molecules of substrate- and productoligopeptides. The inhibition activity in the HIV-1 protease reaction was calculated as follows: % inhibition = 100(A$_{control}$ − A$_{sample}$)/(A$_{control}$); which A is a relative peak area of the hydrolysate. Activity = Area product/(Area substrate + Area product).

Extraction and isolation of phenolic compounds – The dried and powdered aerial parts (1.6 kg) of *O. japonicus* was refluxed with MeOH. This extract (250 g) has been partitioned with organic solvents of the different polarities to afford dichloromethane (80 g), ethyl acetate (29 g), *n*-butanol (37 g) and aqueous (70 g) fractions, respectively. The ethyl acetate fraction (27 g) was subjected to chromatograph using silica gel with CHCl$_3$-MeOH-H$_2$O (25:7:5, lower layer; 7:3:1, lower layer; 65-35-10, lower layer) as solvents

to give E-1~E-36 subfractions (volume of each tube: 30 ml, tubes 1-482). We isolated purely compound A (5, 88 mg) from subfr. E-2 (tubes 6-8) and compound B (1, 43 mg) from the mother layer of subfr. E-2, compound C (4, 88 mg) from E-4 (tubes 14-16), compound D (2, 116 mg) from subfr. E-7 (tubes 28-42), compound E (3, 1879 mg) from subfr. E-12 (tubes 69-135), compound F (7, 20 mg) from the mother layer of subfr. E-14 (tubes 145-174), compound F (8, 27 mg) from subfr. E-18 (tubes 236-237), compound G (7, 16 mg) from subfr. E-23 (tubes 307-308), compound H (6, 13 mg) from the mother layer of subfr. E-23, and compound I (9, 536 mg) from subfr. E-25 (tubes 318-334), respectively. The IR spectra were determined in KBr tablets on a Hitachi 2703 spectrophotometer and the NMR spectra were recorded with a Bruker AM-200 spectrometer.

4-Hydroxybenzoic acid (1) – IRv$_{max}^{KBr}$ cm^{-1}: 3355, 1674, 1608, 1595, 1508, 1448, 1419, 1317, 1290, 1168; ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 7.79 (2H, d, J = 8.6 Hz, H-2 and 6), 6.82 (2H, d, J = 8.6 Hz, H-3 and 5); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 167.3 (C-7), 161.7 (C-4), 131.7 (C-2 and 6), 121.5 (C-1), 115.4 (C-3 and 5).

3,4-Dihydroxybenzoic acid (2) – IRv$_{max}^{KBr}$ cm^{-1}: 3340, 1678, 1600, 1529, 1465, 1419, 1299, 1253, 1190, 1130; ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 7.32 (1H, d, J = 2.1 Hz, H-2), 7.28 (1H, dd, J = 2.1 and 8.2 Hz, H-6), 6.77 (1H, d, J = 8.2 Hz, H-5); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 167.3 (C-7), 150.0 (C-4), 144.9 (C-3), 121.9 (C-1), 121.6 (C-6), 116.5 (C-2), 115.1 (C-5).

Gallic acid (3) – IRv$_{max}^{KBr}$ cm^{-1}: 3345, 1701, 1616, 1425, 1386, 1326, 1263, 1197, 1026; ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 6.92 (2H, s, H-2 and 6); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 167.6 (C-7), 145.6 (C-3 and 5), 138.1 (C-4), 120.6 (C-1), 108.9 (C-2 and 6).

Methyl gallate (4) – IRv$_{max}^{KBr}$ cm^{-1}: 3322, 1697, 1618, 1438, 1382, 1315, 1253, 1197, 1037, 1004; ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 6.93 (2H, s, H-2 and 6), 3.73 (3H, s, COOCH$_3$); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 166.3 (C-7), 145.6 (C-3 and 5), 138.4 (C-4), 119.3 (C-1), 108.5 (C-2 and 6), 51.6 (-CH$_3$).

Kaempferol (5) – ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 8.03 (2H, d, J = 8.8 Hz, H-2' and 6'), 6.92 (2H, d, J = 8.8 Hz, H-3' and 5'), 6.43 (1H, d, J = 1.8 Hz, H-8), 6.18 (1H, d, J = 1.8 Hz, H-6); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 175.9 (C-4), 163.9 (C-7), 160.7 (C-5), 159.2 (C-4'), 156.2 (C-9), 146.8 (C-2), 135.6 (C-

3), 129.5 (C-2' and 6'), 121.7 (C-1'), 115.4 (C-3' and 5'), 103.0 (C-10), 98.2 (C-6), 93.5 (C-8).

Quercetin (6) – ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 7.66 (1H, d, J = 2.1 Hz, H-2'), 7.53 (1H, dd, J = 2.1 and 8.5 Hz, H-6'), 6.87 (1H, d, J = 8.5 Hz, H-5'), 6.40 (1H, d, J = 2.0 Hz, H-8), 6.18 (1H, d, J = 2.0 Hz, H-6); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 175.8 (C-4), 163.8 (C-7), 160.7 (C-5), 156.1 (C-9), 147.7 (C-4'), 146.8 (C-2), 145.0 (C-3'), 135.7 (C-3), 121.9 (C-1'), 119.9 (C-6'), 115.6 (C-5'), 115.0 (C-2'), 103.0 (C-10), 98.1 (C-6), 93.3 (C-8).

Kaempferol 3-*O*-β-D-galactoside (7) – ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 8.06 (2H, d, J = 8.7 Hz, H-2' and 6'), 6.85 (2H, d, J = 8.7 Hz, H-3' and 5'), 6.43 (1H, d, J = 1.7 Hz, H-8), 6.20 (1H, d, J = 1.7 Hz, H-6), 5.40 (1H, d, J = 7.5 Hz, anomeric H); ^{13}C-NMR (DMSO-d$_6$, 50.3 MHz) δ: 177.6 (C-4), 164.2 (C-7), 161.2 (C-5), 160.0 (C-4'), 156.4 (C-2 and 9), 133.4 (C-3), 131.0 (C-2' and 6'), 120.9 (C-1'), 115.1 (C-3' and 5'), 104.0 (C-10), 101.7 (C-1"), 99.4 (C-6), 93.7 (C-8), 75.6 (C-5"), 73.1 (C-3"), 71.2 (C-2"), 67.9 (C-4"), 60.2 (C-6").

Kaempferol 3-*O*-β-D-glucoside (8) – ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 8.04 (2H, d, J = 9.0 Hz, H-2' and 6'), 6.88 (2H, d, J = 9.0 Hz, H-3' and 5'), 6.42 (1H, d, J = 2.0 Hz, H-8), 6.20 (1H, d, J = 2.0 Hz, H-6), 5.44 (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), 159.9 (C-4'), 156.2 (C-2 and 9), 133.2 (C-3), 130.8 (C-2' and 6'), 120.9 (C-1'), 115.1 (C-3' and 5'), 104.0 (C-10), 101.0 (C-1"), 98.7 (C-6), 93.6 (C-8), 77.4 (C-5"), 76.4 (C-3"), 74.2 (C-2"), 69.9 (C-4"), 60.9 (C-6").

Quercetin 3-*O*-β-D-glucoside (9) – ^{1}H-NMR (DMSO-d$_6$, 200 MHz) δ: 7.59 (1H, d, J = 2.0 Hz, H-2'), 7.56 (1H, dd, J = 9.0 and 2.0 Hz, H-6'), 6.83 (1H, d, J = 9.0 Hz, H-5'), 6.39 (1H, d, J = 1.8 Hz, 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-3), 121.6 (C-6'), 121.1 (C-1'), 116.2 (C-5'), 115.2 (C-2'), 104.0 (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").

Results and Discussion

HIV possesses some enzymes that work on 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. 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 cleavage 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. The blocking of any of these steps in the viral life cycle is expected to stop the viral replication. Recently, clarification on the structure and function of viral enzyme, a protease, has shown another target in HIV. Therefore HIV-protease has been a good tool for this investigation since its inhibitors may suppress the HIV-1 production from chronically infected cells (Mcquade *et al.*, 1990; Meek *et al.*, 1989). HIV protease is a homodimer formed from two identical monomers, of 99 amino acid residues each, which possesses a residue of Asp-Thr-Gly as the catalytic site (Tomasselli *et al.*, 1991).

O. japonicus has been used in folk medicine as an anti-cancer agents. In the present study the inhibitory effect against HIV-1 protease from *O. japonicus* and phytochemical study were investigated. The protease inhibitory activity was determined by incubating the extract in a reaction mixture containing protease and His-Lys-Ala-Arg-Val-Leu-(pNO$_2$-Phe)-Glu-Ala-Nle-Ser-NH$_2$ to perform proteolytic cleavage reaction. The cleaved product was measured by reverse-phase HPLC, using a gradient of acetonitrile/0.1% trifluoroacetic acid as a mobile phase. The water extract of *O. japonicus* showed a inhibitory effect on HIV-1 protease by 48.2% at a concentration of 100 μg/ml (Table 1).

The extract of aerial parts of *O. japonicus* was fractionated with dichloromethane, ethyl acetate, n-butanol and water successively. Column chromatography of ethyl acetate soluble fraction afforded nine compounds. Compounds 1 and 2 displayed the presence of hydroxyl, carboxyl and double bond absorptions in its IR spectrum. The ^{1}H-NMR spectrrum

Table 1. Inhibitory effect of *Orostachys japonicus* on the HIV-1 protease

Sample	Inhibition (%)
Water extract	48.2±3.7

The result is the mean±S.D. of 3 replications.

of compound 1 indicated the presence of aromatic signals of ortho coupled doublets at $\delta 6.82$ (2H, J = 8.6 Hz) and $\delta 7.79$ (2H, J = 8.6 Hz), respectively assignable to H-3 and 5 and H-2 and 6. Its ^{13}C-NMR spectrum also showed the signal of oxygen-bearing aromatic ring ($\delta 161.7$) and a ketone group ($\delta 167.3$). Comparison with literature data (Pouchert and Behnkee, 1993) confirmed that compound 1 is 4-hydroxybenzoic acid. The ^{1}H-NMR spectrum of compound 2 indicated the presence of aromatic signals of an ABX type at $\delta 6.77$ (J = 8.2 Hz), 7.28 (J = 2.1 and 8.2 Hz) and $\delta 7.32$ (J = 2.1 Hz), respectively assignable to H-5, H-6 and H-2. Its ^{13}C-NMR spectrum also showed the signals of two oxygen-bearing aromatic ring ($\delta 150.0$, 144.9) and a ketone group ($\delta 167.3$). Compound 2 is characterized as 3,4-dihydroxybenzoic acid. The identity with 3,4-dihydroxybenzoic acid was identified by comparison of ^{1}H- and ^{13}C-NMR spectra with those reported literature (Pouchert and Behnkee, 1993). Compounds 3 and 4 displayed hydroxyl, carboxyl and double bond absorptions in its IR spectrum. The ^{1}H-NMR spectrum of compounds 3 and 4 showed one singlet at $\delta 6.92$ (2H), and two singlets at $\delta 6.93$ (2H) and $\delta 3.73$ (3H) attributable to galloyl and methoxyl protons, respectively. A comparison of the ^{13}C-NMR spectrum of compounds 3 and 4 with literature data (Park et al., 1993) showed it to be gallic acid and methyl gallate, respectively. Compounds 5 and 6 were identified as a well known compound, kaempferol and quercetin by comparison of reported NMR data (Markham et al., 1978). The ^{1}H-NMR spectra of compounds 7-9 showed one anomeric proton signal at $\delta 5.40$ (J = 7.5 Hz), 5.44 (J = 7.0 Hz) and 5.46 (J = 7.0 Hz), respectively. The ^{1}H-NMR spectra of compounds 7 and 8 showed two ortho-coupled doublets ascribable to H-2', 6' and H-3', 5' of B-ring in the flavonoid skeleton and two meta-coupled doublets ascribable to H-8 and H-6 of A-ring in the flavonoid skeleton. However the ^{1}H-NMR spectra of compound 9 showed an ortho-coupled doublet, a meta-coupled doublet and a ortho, meta-coupled doublet-doublets attributable to H-2', H-6' and H-5' of B-ring, respectively. Compounds 7-9 gave a positive reaction in Molisch test. The sugar moiety of compounds 7 and 8-9 were determined to be β-D-galactopyranose and β-D-glucopyranose, respectively, by the J values of the anomeric proton signals and the ^{13}C-NMR data. The ^{13}C-NMR data of these compounds supported the attachment of sugar moiety to the C-3 position of flavonoid glycosides.
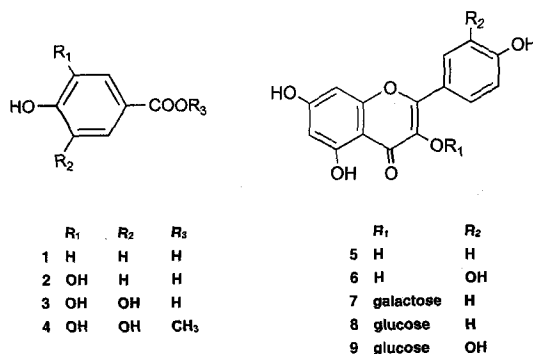

Fig. 1. The compounds isolated from the aerial parts of *Orostachys japonicus*. 1: 4-hydroxybenzoic acid, 2: 3,4-dihydroxybenzoic acid, 3: gallic acid. 4: methyl gallate, 5: kaempferol, 6: quercetin, 7: kaempferol 3-*O*-β-D-galactoside, 8: kaempferol 3-*O*-β-D-glucoside, 9: quercetin 3-*O*-β-D-glucoside.

From the above results, compounds 7, 8 and 9 were characterized as kaempferol 3-*O*-β-D-galactoside, kaempferol 3-*O*-β-D-glucoside and quercetin 3-*O*-β-D-glucoside, respectively. 4-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, gallic acid, methyl gallate and kaempferol 3-β-D-galactoside were the first report of the isolation from this plant. Investigation of anti-HIV protease components from *O. japonicus* is now in progress.

Acknowledgements

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