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# Screening of Some Indonesian Medicinal Plants for Inhibitory Effects on HIV-1 Protease

INES TOMOCO KUSUMOTO,[a] NOBUKO KAKIUCHI,[a] MASAO HATTORI,*,[a]
TSUNEO NAMBA[a], SUPRIYATNA SUTARDJO[b] and KUNITADA SHIMOTOHNO[c]

[a] *Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University, 2630, Sugitani, Toyama 930-01, Japan*
[b] *Department of Pharmacy, Padjadjaran University, Jatinangor, Sumedang 45363, Indonesia*
[c] *Virology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo 104, Japan*

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Methanol and water extracts of 30 Indonesian plants were investigated for their inhibitory activity on HIV-1 protease (PR). The PR inhibitory activity was determined by incubating the extracts in a reaction mixture containing PR and substrate His-Lys-Ala-Arg-Val-Leu-(p$NO_2$-Phe)-Glu-Ala-Nle-Ser-$NH_2$ at pH 5.0 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. Of the extracts tested, the methanol extracts of *Swietenia mahagoni* (bark) and *Terminalia belerica* (fruit peel) and both methanol and water extracts of *Woodfordia floribunda* (flower and leaves) showed a strong PR inhibition, with $IC_{50} \leqq 50$ $\mu$g/ml. Five other samples showed more than 80% inhibition at 0.5 mg/ml.

**Keywords**——HIV-1 protease; protease inhibitors; Indonesian plants

Proteases (PR) of retrovirus, especially human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), have been demonstrated to play an essential function in the viral replication.[1] In HIV-1, the gag-pol genes are to be translated to the respective polyproteins which have to be cleaved in certain specific sites by protease to produce essential viral enzymes such as reverse transcriptase, ribonuclease, endonuclease, the protease itself and structural proteins of mature virions, for example, p17, p9, p7 and p24. The HIV-1 PR of molecular weight 11,000 daltons is an aspartic protease containing an Asp-Thr-Gly residue as its active site. Since this enzyme seems to provide a promissing target for specific anti-viral drugs, methods of analysis of PR activity by enzyme assay have been established, in which peptides having the amino acid sequences of the polyprotein, such as gag p17/p24, gag p24/p7, prot/RT are used as substrates.[2,3] Improvements of the monitoring system of the proteolytic cleavage by spectrophotometric analysis[4-6] or by radiometric assay[7,8] have been reported, and several types of peptide HIV-PR inhibitors,[2,9,10] and non-peptide inhibitors, cerulenin[11] and hydroxyhaloperidol[12] have also been reported.

To find out naturally-occurring substances which may function as a potent and specific inhibitor of HIV-1 PR, we examined some Indonesian plants used in the traditional medicinal system "Jamu" for their PR inhibitory activities, by determining the proteolytic activity of PR by HPLC. Some plants were found to be effective inhibitors of a recombinant HIV-1 PR.

## Materials and Methods

**Indonesian plants**——Plant materials were purchased from a supplier of Jamu materials in Bandung, Indonesia. Water and methanol extracts were prepared by refluxing 5.0 g of material in water or methanol (100 ml×2) for 3 h, and then removing the solvents under reduced pressure.

**Peptides and chemicals**——The peptide having an aminoacid sequence corresponding to the p24 -p15 cleavage site, *i.e.*, His-Lys-Ala-Arg-Val-Leu-(p$NO_2$-Phe)-Glu-Ala-Nle-Ser-$NH_2$ was obtained from Peptide Institute, Inc. (Osaka, Japan). A stock solution of the peptide (2.0 mg/ml) was prepared in 50 mM NaOAc (pH 5.0). Tryptone and Yeast extract were purchased from Difco Laboratories (Detroit, MI, USA), and ampicillin, lysozyme

and phenyl methyl sulfonyl fluoride (PMSF) from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

**Preparation of bacterial protein**——The construction of the bacterial expression system will be described elsewhere. *Escherichia coli* JM105 cells bearing puEC vector of the DNA sequence of HIV-1 PR were grown overnight at 30°C in 5 ml of Luria broth (10 g of trypton, 5 g of yeast extract and 10 g of NaCl in 1.0 *l* solution) containing ampicillin at 100 $\mu$g/ml. The overnight culture was transfered to 400 ml of Luria broth and the cells were shake-cultured at 30°C to $OD_{600}=0.6$. Then, the culture was incubated for another 3 h at 42°C for the induction of the PR expression. The cells were collected by centrifugation at $4,000\times g/15$ min and the pellets were suspended in 20 ml of solution A (50 mM phosphate buffer, pH 7.8 containing 300 mM NaCl, 1 mM PMSF and 5 mg of lysozyme), kept in ice water for 5 min and then disrupted by sonication for 10 min at 4°C. The suspension was then subjected to centrifugation at $15,800\times g/10$ min and the pellets obtained were solubilized in 6 M guanidine-HCl. The guanidine solution was dialyzed two times against buffer 1 (25 mM NaOAc solution, pH 5.0, containing 4 M urea, 10% glycerol, 5% ethyleneglycol, 1 mM dithiothreitol (DTT) and 0.2% Nonidet P-40) then, two times against buffer 2 (25 mM NaOAc solution, pH 5.0, containing 2 M urea, 50% glycerol, 0.2% Nonidet P-40 and 1 mM DTT) and then stored at −85°C. The presence of HIV-1 PR in the bacterial extract was demonstrated by the peptide band of 15,000 daltons in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel). Aliquots of the dialyzed solution were diluted in buffer 3 (50 mM NaOAc solution, pH 5.0, containing 1.0 mM DTT, 20% glycerol, 10% ethyleneglycol and 0.3% bovine serum albumin) before assaying. One unit of HIV-1 PR activity was arbitrarily defined as a quantity of the enzyme capable of cleaving 1.0 $\mu$mol of the substrate in 10 min at 37°C at pH 5.0.

**Enzyme assay**——To a reaction mixture (10 $\mu$l) of 50 mM acetate buffer (pH 5.0) containing 8.0 $\mu$g of the substrate and 1.0 $\mu$l of a plant extract solution in distilled water or dimethylsulfoxide (DMSO; the final concentration of DMSO was 10% in the reaction mixture), 25 units of HIV-1 PR was added and the mixture was incubated at 37°C. Two hours later, the reaction was stopped by addition of 13 $\mu$l of 0.5% trifluoroacetic acid (TFA) and the

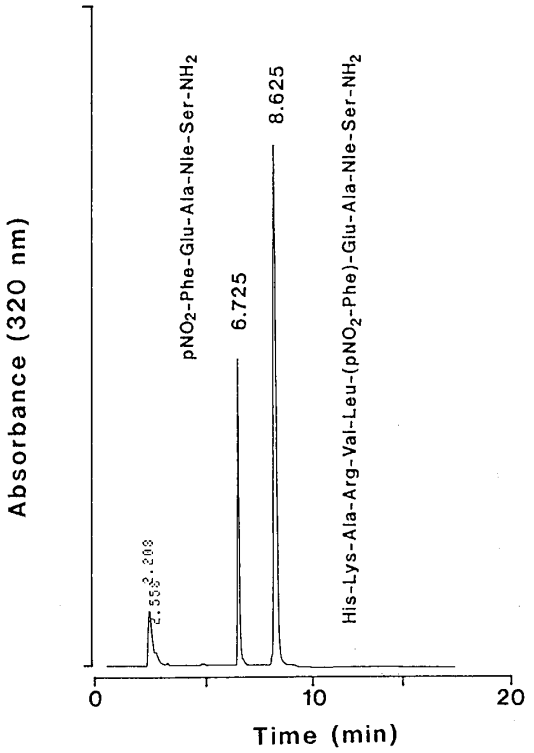


Fig. 1. HPLC Profile of a Reaction Mixture of HIV-1 Protease and Substrate Incubated for 2 h at 37°C
After the addition of 0.5% TFA to stop the reaction, the reaction mixture was applied to a reverse-phase HPLC with a gradient of acetonitrile in 0.1% TFA, as described in "Materials and Methods." The retention times of the substrate itself and one of the hydrolysed products were 8.6 min and 6.7 min, respectively.

TABLE I. Effect of Indonesian Plants on HIV-1 Protease Activity

| Sample No. | Local name | Botanical name | Parts | $IC_{50}$ (μg/ml) | |
|---|---|---|---|---|---|
| | | | | MeOH ext. | $H_2O$ ext. |
| 1 | Akar usar | *Andropogon zizanioides* (L.) URBAN | root | 500 | >500 |
| 2 | Adas | *Foeniculum vulgare* MILL. | seed | 100 | >500 |
| 3 | Anyang | *Elaeocarpus grandiflorus* SMITH | fruit | 100 | 100 |
| 4 | Benalu teh | *Loranthus parasiticus* (L.) MERR. | stems+branches | 100 | 260 |
| 5 | Benalu teh | *Loranthus parasiticus* (L.) MERR. | whole plant | 240 | 100 |
| 6 | Bidara laut | *Strychnos nux-vomica* L. | bark | 500 | >500 |
| 7 | Cecendet | *Physalis angulata* L. | aerial part | 340 | >500 |
| 8 | Congkok | *Curculigo orchioides* GAERTN. | fruit | 400 | 400 |
| 9 | Gadung | *Dioscorea hispida* DENNST. | rhizome | >500 | >500 |
| 10 | Ganitri | *Elaeocarpus sphaericus* (GAE.) K. SCHUM. | fruit | >500 | 220 |
| 11 | Jelawe | *Terminalia belerica* ROXB. | fruit peel | 50 | 220 |
| 12 | Kayu angin | *Usnea misaminensis* VAIN. | whole plant | 220 | >500 |
| 13 | Kayu rapat | *Parameria laevigata* MOLDENKE. | bark | 100 | >500 |
| 14 | Kecibeling | *Strobilanthes crispus* L. | leaves | >500 | >500 |
| 15 | Kedawung | *Parkia roxburghii* BENTH. | seed | >500 | >500 |
| 16 | Kikoneng | *Ficus edelfelitii* KING | bark | >500 | >500 |
| 17 | Kiules | *Helicteres isora* L. | fruit | 380 | 500 |
| 18 | Mahoni | *Swietenia mahagoni* L. | bark | 40 | 100 |
| 19 | Manggis | *Garcinia mangostana* L. | peel | 50 | 100 |
| 20 | Pulasari | *Alyxia reinwardtii* BL. | bark | >500 | >500 |
| 21 | Saga | *Abrus precatorius* L. | seed | >500 | >500 |
| 22 | Saga pohon | *Adenanthera pavonina* L. | seed | >500 | >500 |
| 23 | Secang | *Caesalpinia sappan* L. | bark | 280 | 320 |
| 24 | Sedawa | *Woodfordia floribunda* SALISB. | flower+leaves | 50 | 50 |
| 25 | Sambiloto | *Andrographis paniculata* NEES. | leaves | 500 | 500 |
| 26 | Sintok | *Cinnamomum sintok* BL. | bark | 220 | 320 |
| 27 | Supratul | *Sindora sumatrana* MIQ. | fruit | 260 | 360 |
| 28 | Tapak leman | *Elephantopus scaber* L. | whole plant | 500 | >500 |
| 29 | Temu ireng | *Curcuma aeruginosa* ROXB. | rhizome | 500 | >500 |
| 30 | Temu lawak | *Curcuma xanthorriza* ROXB. | rhizome | 300 | >500 |

hydrolysate was analyzed by HPLC. During the incubation, 50% of the substrate was hydrolyzed at the site of Leu and ($pNO_2$)-Phe, and the *K*m value obtained in this condition was 166 μM. As an inhibitor control, 0.1 μg of acetylpepstatin (Bachem Feinchemikalien AG, Bubendorf, Switzerland) was used, whose percentage of inhibition was *ca.* 50%.

**HPLC**——A LC9A liquid chromatograph and a SPC-6A UV spectrophotometric detector (Shimadzu Corporation, Kyoto, Japan) were used. Ten μl of the reaction mixture was injected by an auto injector (Shimadzu SIL-6B) into a LiChrospher 100 RP-18 column (250×4 mm, Merck, Darmstadt, FRG) which was eluted with a gradient of acetonitrile (20–50%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min, and 40°C. The elution profile was monitored at 320 nm. The substrate and Phe($NO_2$)-bearing hydrolysate were eluted at 8.6 and 6.7 min, respectively (Fig. 1). The PR activity was calculated from the ratio of the substrate peak area to the product peak area by using an integrator C-R6A Chromatopac (Shimadzu).

## Results and Discussion

Methanol and water extracts of 30 Indonesian plants were examined for their inhibitory effects on the HIV-1 PR activity. TABLE I shows the plants with their local and botanical names and their inhibitory activities expressed as the concentrations of extracts that inhibited 50% of the enzyme activity ($IC_{50}$), in μg/ml. The methanol extracts of **11**, **18**, **19** and **24** and the water extract of **24** showed very

potent inhibitory activities with $IC_{50} \leqq 50$ $\mu$g/ml. The methanol extracts of **2**, **3**, **4** and **13** and water extracts of **3**, **5**, **18** and **19** showed a significant inhibition, with $IC_{50}$ of 100 $\mu$g/ml. The methanol extracts of **5**, **23**, **26** and **27** and the water extract of **10**, at a concentration of 500 $\mu$g/ml, inhibited more than 80% of the protease activity. To identify the active principle contained, one of the potent inhibitors, the methanol extract of **18** was further fractionated into ethylacetate-, butanol- and water-soluble fractions, and these fractions (each 50 $\mu$g/ml), were tested for their PR inhibitory activity to give 34, 67 and 74% of inhibition, respectively. Further studies on the isolation of the inhibitor are now in progress.

Protease have been the focus of biochemical and pharmacological research as they are known to function as a trigger of many vital reactions. Intense studies have been made in this field. Several proteases have newly been discovered in microorganisms and humans, and the specific functions and structures of some viral proteases, such as HIV-1 and -2, HTLV-1, AMV, MLV and other retroviruses PR[13] have been reported.

In the previous paper, we reported that some Indonesian plant extracts have inhibitory action on RNA-tumor virus reverse transcriptase (RT), which is an enzyme functioning to produce a double strand DNA from viral RNA during the viral replication[14] and therefore considered to be a suitable target for the development of anti-HIV agents. The extracts used in the present experiment showed RT inhibitory activity as well as PR inhibition. However, the methanol extracts of **2** and **13** and the water extracts of **3**, **18** and **19** were found to be far more potent as PR inhibitors, which suggested the presence of selective inhibitors acting on HIV-1 PR in these samples.

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