PURIFICATION AND CHARACTERIZATION OF ANTI-HIV-1 PROTEIN FROM *CANNA INDICA* L. LEAVES

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Abstract. A novel 10 kDa protein with anti-HIV-1 reverse transcriptase (RT) inhibitory activity was isolated from leaves of *Canna indica* L. using a combination of native-PAGE and ammonium sulfate precipitation. HIV-1 and RT inhibitory activity was measured using a syncytium forming $^{\Delta Tat/Rev}$ MC99 virus in Tat/Rev transfected 1A2 cell line and ELISA technique, respectively. Edman N-terminal and internal amino acid sequence (using LC-MS-MS) determination revealed the 10 kDa *Canna indica* L. leaf protein as a putative plastocyanin. This is the first report of a plant plastocyanin with HIV-1 RT inhibitory property.

Keywords: *Canna indica* L., anti-HIV-1 reverse transcriptase inhibitory activity, syncytium reduction assay, plastocyanin

INTRODUCTION

Plants are promising sources of novel compounds that possess anti-HIV activity. They include proteins and peptides, *viz*. macrocyclic peptide lectin from *Myrianthus holstii* Engl. (Charan *et al*, 2000) and a plant protein, MRK29, from Thai bitter gourd (Jiratchariyakul *et al*, 2001), both having an inhibitory effect on HIV reverse transcriptase (RT) *in vitro*. More recently, HIV-1 RT inhibitory activity have been reported from Kunitz type trypsin inhibitor (KBTI) purified from Korean large black soybean (Fang *et al*, 2010), acaconin extracted from the seeds of *Acacia confusa* Merr. (Lam and Ng, 2010), proteins of 31 kDa and 14 kDa from *Canna indica* L. rhizomes (Woradulayapinij *et al*, 2005). BanLec, a jacalin-related lectin isolated from banana fruit inhibits HIV-1 through binding to HIV-1 envelope glycoprotein, gp120 and blocks entry of the virus into host cell (Swanson *et al*, 2010).

Canna indica L. is a tropical herb belonging to the family Cannaceae. It has been widely used in traditional medicine as diaphoretic and diuretic in treating fever and dropsy (Duke and Ayensu, 1985). Its decoction extract shows antitumor activity in rat, weak cytotoxic activity towards cell culture (Pornsiriprasert *et al*, 1986) and antibacterial activity (Avirutnant and Pongpan, 1983).

In this study, we purified a protein

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from *Canna indica* L. leaves that exhibited anti-HIV-1 RT inhibitory activity and inhibited the defective $^{\Delta Tat/Rev}$ MC99 virus using syncytium reduction assay.

MATERIALS AND METHODS

Plant extraction

Canna indica L. was collected from the Medicinal Plant Siri-Ruckhachati Garden, Faculty of Pharmacy, Mahidol University at Salaya Campus, Nakhon Pathom Province, Thailand. It was identified by Professor Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University.

Roots, rhizomes and leaves were separately sliced, crushed into small pieces and blended with cold sterile distilled water. The crude extracts were filtered through cheese-cloth and centrifuged at 14,240*g* for 10 minutes and filtered through Whatman no.1 paper. The clear supernatant was fractionated by precipitation with 30, 60 and 80% ammonium sulfate, respectively (Harris, 1989). The protein fractions were resuspended in sterile distilled water containing 1 mM EDTA and dialyzed (MWCO 6-8000 Da cut-off, Spectrum Medical Industries, Los Angeles, CA) overnight at 4ºC against four changes of 3 liters of distilled water containing 1 mM EDTA. Then, the protein fractions were filtered through cellulose acetate membrane (0.45 µm pore size), lyophilized and stored at -20ºC until assayed.

Protein purification

Protein fractions were separated by 15% native-PAGE in 1 M Tris-HCl buffer (pH 8.8) containing 40% glycerol, 0.05% bromphenol blue, and 10% 2-mercaptoethanol at constant 100 V at 4º**C.** Protein bands were stained with 0.1% Coomassie blue dye, destained with 15% methanol containing 7% glacial acetic acid, and the protein bands of interest were eluted with Tris-glycine buffer (0.3% Tris-base, 1.44% glycine, dissolved in double distrilled water) using Electro-Eluter (Bio-Rad, Hercules, CA) at 10 mA for 3 hours (Hames, 1990). The eluted proteins were dialyzed against distilled water containing 1mM EDTA and concentrated by an Ultrafree centrifugal filter device (Millipore, Billerica, MA), lyophilized, then stored at -20ºC until assayed. Protein concentration was determined using Bradford method (Bradford, 1976).

N-terminal protein sequencing

After 15% SDS-PAGE, the purified protein was electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Mini Trans-Blot® Electrophoretic Transfer cell; Bio-Rad, Hercules, CA) using a transferring buffer of 10 mM CAPS and 10% (v/v) methanol pH 11.0. Then, it was briefly stained with 0.1% Coomassie blue R-250 for visualizing the protein band and subjected for the N-terminal sequencing by Edman degradation method (Interdisciplinary Center for Biotechnology Research; ICBR Protein core, University of Florida and Central (Laboratory, Faculty of Agriculture Science, Mie University, Japan).

Peptide sequencing by mass spectrometry (MS)

In order to prepare protein samples for internal amino acid sequencing by MS, the purified protein was separated by 2-D electrophoresis [isoelectrofocusing (pH 3-10) followed by 12% SDS-PAGE] and stained briefly with Coomassie brilliant blue R-250 to locate protein spots. The gel was destained completely, washed with distilled water and target protein spot was excised and subjected to in-gel tryptic di-

gestion (Shevchenko *et al*, 2006). The tryptic peptides were sequenced by nanoLC-RSI-MS/MS (Central Instrument Facility, Faculty of Science, Mahidol University). The MS/MS data were subjected to Mascot Protein Search Database (MSDB) search engine (www.matrixscience.com) for protein identification under the Viridiplantae (green plants) taxonomy. The peptide mass tolerance and fragment mass (MS/MS) tolerance allowed was ± 1.2 Da and ± 0.6 Da, respectively. To identify putative functions and characteristics of identified proteins, SwissProt (www.expasy.org) and NCBI (www.ncbi.nlm.nih.gov) were used.

Cell-based assay for anti-HIV-1 property

The ability of extracts to inhibit syncytium formation of 1A2 cells derived from CEM-SS (human T-cell lymphoblastic leukemia) infected with $^{\Delta Tat/Rev}$MC99 virus, which is incapable of replicating in peripheral blood mononuclear cells and integrating into host genome by reverting to wild type (Chen *et al*, 1992), was determined in duplicate in 96-well tissue culture plates. Cells were treated with 10 µl/ml polybrene, and then incubated with $^{\Delta Tat/Rev}$MC99 in order to obtain a virus titer of 100-200 syncytium forming unit (SFU)/50 µl. The 1A2 cells were suspended ($5x10^5$ cells/ml) and seeded at $5x10^4$ cells/100 µl/well that contained 50 µl of medium (RPMI 1640 plus 10% fetal calf serum and 1 µg/ml gentamicin) and 50 µl of 2-fold dilutions of lyophilized extracts dissolved in medium, in triplicate. Azidothymidine (AZT) was used as positive control. Negative controls included cells that contained neither the azidothymidine nor virus, uninfected cells treated with the corresponding dilutions of extract only and virus-infected cells. After incubation for 3 days at 37ºC under an atmosphere of 5% CO_2, the numbers of syncytia were counted under a light microscope. Anti-HIV-1 activity is expressed as the concentration of the plant extract that inhibits syncytium formation by 50% of infected cell control (EC_{50}).

Cytotoxicity assay procedure was similar to the syncytium inhibition assay, except that after incubation at 37ºC for 3 days, 50 µl aliquot of tetrazolium salt (1 mg/ml) (XTT; Boehringer Mannheim, Germany) containing 1% phenazine methosulfate (Sigma, St Louis, MO) was added per well. After incubation for 3 hours, viable cells produced a yellow-brown color of formazan product. The optical densities (OD) at 450 nm and 650 nm were measured. Results of the average OD from test (T) and control (C) wells are recorded as percent cell viability, using the formula [(OD test – OD extract control)/(OD cell control– OD medium control)] x 100, and expressed as the concentration that inhibited 50% of cell viability (IC_{50}). Anti-HIV-1 results are expressed in terms of therapeutic index (TI), namely, (IC_{50})/(EC_{50}).

HIV-1 reverse transcriptase inhibitory assay

HIV-1 reverse transcriptase inhibitory assay was performed using a colorimetric enzyme immunoassay to measure incorporation of biotinylated digoxigenin-labeled dUMP into DNA. The commercial assay kit, version 13.0, was purchased from Roche, Germany. The reaction mixture (60 µl) composed of 46 mM Tris-HCl, 226 mM KCl, 27.5 mM $MgCl_2$, 9.2 mM DDT, 10 µM dUTP/dTTP, template/primer duplex (0.75 A_{260}/ml), 10 mU recombinant HIV-1-RT, and dilutions of the plant extract dissolved in sterile distilled H_2O was incubated at 37ºC for 1 hour. Controls included reactions without the plant extracts [replaced with 1 mM doxo-

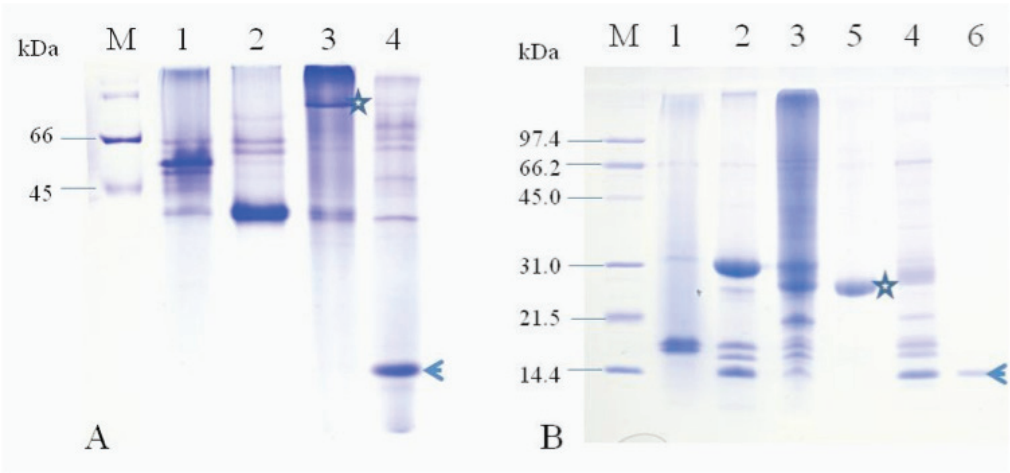

Fig 1–Native-PAGE (A) and SDS-PAGE (B) of *Canna indica* L. Gels were stained with Coomasie blue dye. Lane M, protein marker; lane 1, root; lane 2, rhizome; lane 3, leaf extract precipitated with 30-60% ammonium sulfate; lane 4, leaf extract precipitated with 60-80% ammonium sulfate; lane 5, protein band (☆) eluted from lane 3 of native-PAGE; lane 6, protein band (◄) eluted from lane 4 of native-PAGE.

rubicin (Sigma)] and RT as the positive. Then the plates were washed with 250 µl of washing buffer, and a 200 µl aliquot of anti-digoxigenin-peroxidase (200 mU/ml) solution was added per well and incubated at 37ºC for 1 hour. Then the wells were washed as described above and 200 µl aliquot of peroxidase substrate solution [2,2-azinobis (3-ethylbenzotiazoline-6-sulfonic acid) diammonium salt] was added and incubated at 25ºC for 10 minutes. Absorbance at 405 nm was measured using a microplate reader (Model 680, Bio-Rad Hercules, CA) (reference wavelength at 490 nm). Inhibition ratio of the tested plant extracts is calculated as follows:

$$(\%)\ IR = 1 - \left[\frac{OD\ (complete\ system + compound) - OD\ (complete\ system - RT)}{OD\ (complete\ system + RT) - OD\ (complete\ system - RT)} \right] \times 100$$

RESULTS

Protein extracts from *Canna indica* L.

The crude proteins of roots, rhizomes and leaves from *Canna indica* L. were separated by native-PAGE and analyzed by SDS-PAGE (Fig 1A and 1B, respectively). The protein content of root was 0.03% (w/w) and that of leaf 0.05% (w/w). Thus, the leaf protein extract was eluted from native-PAGE gel (Fig 1A lane 3 asterisk, lane 4 arrow) and further purified using 30-60% and 60-80% ammonium sulfate precipitation to obtain protein of 30 kDa (Fig 1B lane 5) and 10 kDa (Fig 1B lane 6), respectively, as estimated by SDS-PAGE.

Cell-based assay of anti-HIV-1 activity of *Canna indica* L. proteins

The protein extracts of *Canna indica* L. from roots, rhizomes and leaves were tested for cytotoxic and anti-HIV-1 activity using cell-based assays, namely, XTT of 1A2 cells and syncytium formation of $^{\Delta Tat/Rev}$MC99 virus infected 1A2 cells,

Table 1
Cytotoxic, anti-HIV-1 activity and HIV-1 RT inhibitory activity of crude extracts and purified leaf proteins from *Canna indica* L.

Protein sample	Cell-based assay			anti-HIV-1 RT activity (percent IR[c])
	IC_{50} (μg/ml)	EC_{50} (μg/ml)	TI	
Crude extract				
Leaf: 30-60% ammonium sulfate precipitation fraction	195 ± 77 [a]	7 ± 10 [a]	156 ± 153 [a]	89
Leaf: 60-80% ammonium sulfate precipitation fraction	160 ± 40 [a]	1 ± 1 [a]	307 ± 373 [a]	81
Root	139 ± 8 [b]	1.3 ± 0.8 [b]	133 ± 78 [b]	90
Rhizome	111 ± 5 [b]	1.3 ± 0.7 [b]	96 ± 55 [b]	18
Purified leaf protein				
30 kDa	256 ± 135 [b]	6 ± 5 [b]	49 ± 16 [b]	ND
10 kDa	193 ± 96 [a]	1.2 ± 0.6 [a]	162 ± 73 [a]	94
AZT	$>10^{-8}$ μM	$2.58x10^{-9}$ μM	>3.87	ND
	$>10^{-8}$ μM	$3.52x10^{-9}$ μM	>2.84	ND

[a] Range of doses assayed was 0.025-250 μg/ml; results are mean ± S.E.M. (n = 3).
[b] Range of doses assayed was 0.025-250 μg/ml; results are mean ± S.E.M. (n = 2).
[c] Doses assayed was 50 μg/ml and 1 mM doxorubicin hydrochloride was used as positive control exhibiting 99.9% RT inhibition.
ND, not done

respectively. The results of these assays, expressed in terms of IC_{50}, EC_{50} and TI are summarized in Table 1. Higher TI values were obtained from crude leaf protein extracts compared to root and rhizome. TI value of 10 kDa protein was higher than that of 30 kDa protein but was half of the TI value of the crude extract (60-80 ammonium sulfate precipitate) from which it was purified. All protein fractions, including purified proteins, showed better anti-HIV-1 activity than AZT.

HIV-1 RT inhibitory activity of *Canna indica* L. proteins

Using a commercial RT assay kit, all crude protein extracts (except rhizome) exhibited HIV-1 RT inhibitory effect (expressed as % IR) (Table 1), concordant with the cell-based inhibition results. Thus the 10 kDa protein purified from leaves (60-80% ammonium sulfate precipitation) was subjected to further studies.

Identification of anti-HIV-1 of *Canna indica* L. leaf 10 kDa protein

The 10 kDa protein was subjected to Edman N-terminal sequencing, which showed FDVLLGGDGGVLAIV, 82% identical with the N-terminal sequence of plastocyanin precursor of *Hordeum vulgare* L. (Nielsen and Gausing, 1993) and 83% with plastocyanin of *Solanum tuberosum* L. (Ramshaw *et al*, 1974). The internal peptide sequence determined by MS was GTYSFYCAPHQGAGMVGKV. Using BlastP software, the 10 kDa protein was identified as a putative plastocyanin. Using Expasy-tool, the deduced amino acid sequences of other plastocyanin genes

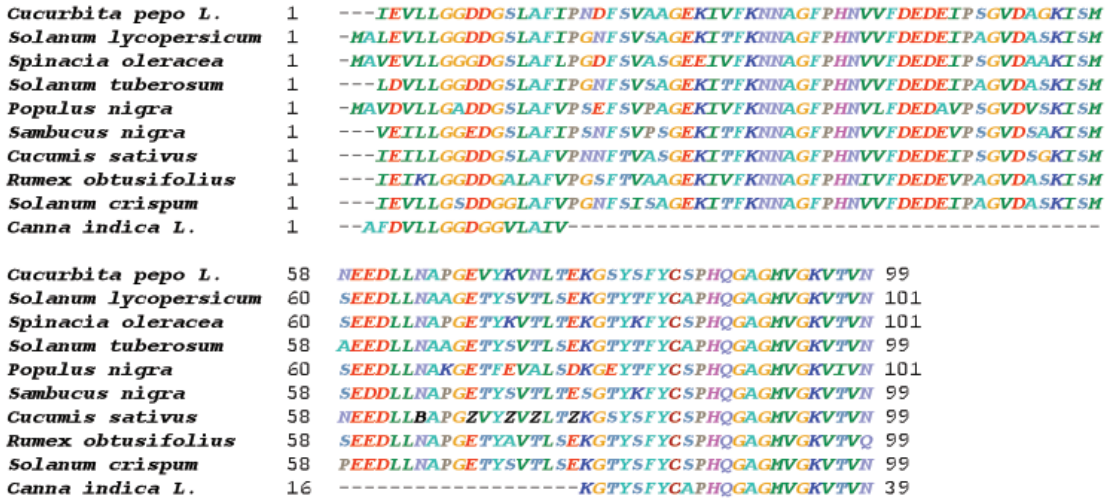

Fig 2–Comparison of N-terminal and internal amino acid sequences of plastocyanins. *Canna indica* L. (this study), *Cucurbita pepo* L. (Scawen and Boulter, 1974), *Solanum lycopersicum* L. (Detlefsen *et al*, 1989), *Spinacia oleracea* L. (Scawen *et al*, 1975), *Solanum tuberosum* L. (Ramshaw *et al*, 1974), *Populus nigra* L. (Freeman, 1979), *Sambucus nigra* L. (Scawen *et al*, 1974), *Cucumis sativus* L.(Ramshaw and Felton, 1982), *Rumex obtusifolius* L. (Haslett *et al*, 1974), *Solanum crispum* Ruiz & Pav (Haslett *et al*, 1978).

containing 100 amino acids deposited in protein database (*Cucumis sativus* L., *Solanum lycopersicum* L., *Spinacia oleracea* L., *Rumex obtusifolius* L., *Sambucus nigra* L., *Solanum tuberosum* L., and *Cucurbita pepo* L) were compared with that of the *Canna indica* L. 10 kDa protein (Fig 2).

DISCUSSION

A cell based assay for HIV-1 inhibitor and HIV-1 RT inhibitory assay were used to test crude protein extracts of *Canna indica* L. All protein samples from leafs, roots and rhizomes showed strong anti- HIV-1 activities using the cell based assay. However, the protein extract from rhizomes exhibited weaker HIV-1 RT inhibitory activity compared to leaf and root extracts. These results are similar to those of Woradulayapinij *et al* (2005).

The proteins from leaves, which exhibited strongest anti-HIV-1 activity, were subjected to purification and characterization. Proteins were purified without using any column chromatography, but separated by native-PAGE, and eluting band of interest. This method has the ability to preserve the physical and biological properties of the protein for further analysis (Eubel *et al*, 2005).

Ammonium sulfate fractionation of leaf protein band eluted from native-PAGE revealed a 10 kDa protein with potent anti-HIV-1 RT inhibitory activity (IC_{50} = 9.3 µg/ml). Amino acid sequencing using Edman N-terminal sequencing revealed the 10 kDa protein as a putative plastocyanin. Plastocyanin is a copper-containing protein involved in electron-transfer (Rienzoa *et al*, 2004). The protein is a monomer of 10.5 kDa, containing 99 amino acids in most vascular plants. Higher-plant plastocyanins are strongly acidic proteins.

This study is the first report of plastocyanin from *Canna indica* L. with property as an anti-HIV-1 RT inhibitor. This novel function needs to be further investigated.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Punchapat Sojikul, Department of Biotechnology, Faculty of Science, Mahidol University for assistance with protein analysis and Dr Talabporn Harnroongroj, Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University for proof reading of the manuscript. This project was supported by a scholarship from the Office of Higher Education Commission Staff Development Project and a Mahidol University research grant.

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