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HIV- 1 Reverse Transcriptase Inhibitors fromThai Medicinal Plants and *Elephantopus scaber* Linn.

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Abstract

Twenty- two selected Thai medicinal plants were extracted with water and 80% ethanol method and tested for their inhibitory effects on the HIV-1 RT by *in vitro* radiometric method. The results showed that eleven water extracts and seven ethanol extracts exhibited the anti-HIV-1 RT activities. Potent HIV-1 RT inhibitory actions were obtained from the water extract of *Jatropha curcas* L. stem barks, water extract of *Elephantopus scaber* L. leaves and root, and ethanol extract of *Securinega virosa* Baill. branches and leaves. They exhibited the percentage of inhibition ratio (% IR) at 97.5, 96.9, and 88.2, respectively. Positive tannin reaction were found from the extracts of *J. curcas* and *S. virosa*, but not found from *E. scaber* extract. Therefore, the water extract of *E. scaber* was selected for further study. IC_{50} of leaves and root were 69.9 and 107.57 µg/ml, respectively. The inhibitory mode of action of *E. scabe*r was non-competitive with respect to the substrate. The water extract of *E. scaber* was fractionated with ammonium sulfate at 30%, 30-60%, and 60-80% saturation. The proteins were separated by sodium dodecyl sulfate acrylamide gel electrophoresis. The interesting protein band was cut-off, eluted, and assayed for the anti-HIV-1 RT activity. Its IC_{50} was approximately 4.29 µg/ml. The molecular weight of the protein was calculated to be 34.5 kDa. Its isoelectric point (pI) was 4.65 and the amino acid sequence of the N-terminus was analyzed to be Alanine-Alanine-Alanine-Glutamine-Proline-Phenylalanine-Glycine-Asparagine.

Key word: *Elephantopus scaber*, Compositae, Anti-HIV-1 reverse transcriptase.

INTRODUCTION

Medicinal plants have been widely studied for their anti-HIV activity. Some plant extracts and compounds were reported as HIV inhibitor, such as a xylanase from roots of sanchi ginseng[1], Flavanone and flavonol glycosides from the leaves of Thevetia peruviana[2], extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia,* and *Elaeophorbia drupifera*[3], peptidic protease inhibitor from Vicia faba seeds[4] , leaf extract of Terminalia triflora.,[5] , deladin from *Delandia umbellate*[6]. *Calophyllum coumarins* and xanthones[7]. MRK29 of *Momordica charantia*[8] were reported as anti-HIV agents. However, there is no medicinal plant can be developed as HIV-I inhibitor drug. For our preliminary study, some Thai medicinal plants which prescribed by folkloric doctors as antiviral medicine were screened for HIV-1 inhibitory activity. Subsequently, the most promising plant, *Elephantopus scaber* L., was selected for further study.

E. scaber L., a member of Family Compositae, is a inconspicuous herb growing throughout Thailand. The plants bear a rosette of basal leaves and the small pink florets are borne on long stalks. It can grow in all tropical regions in the rainy season (But, 1997. In Thailand, there are different local names, such as, Do mai ru lom (Central), Khing fai nok khum (Chaiyaphum), and

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Prickly leaves elephant's foot *E. scaber* has been used as traditional medicine in many countries. In Thailand, it has been used as traditional medicine[9]. In Brazil, the decoction of whole plant is used to stimulate diuresis, reduce fever and eliminate bladder stones[10]. In China[11] and Taiwan[12] are used as a diuretic and anti-febrile, an antiviral, an antibacterial agent, as well as in the treatment of hepatitis, bronchitis, the cough associated with pneumonia, and arthralgia. The antitumor activity of the leaves of *E. scaber* has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice[13]. Hepatpprotection was reported by Rajesh, and Latha[14].

There are many compounds found in *E. scaber* such as, elephantopins and triterpenes. The known compounds that exhibit the cytotoxic, antitumoral and antileukemia activity are sesquiterpene lactones[10], germacranolides and triterpenes[12], molephantin[15], tomenphantopin-a and b[16], and guaianolide glycoside[17]. The leaves contain a lectin that preferentially agglutinates human B group erythrocytes, an uncommon feature.

This research work was emphasized on screening for potent inhibitors of HIV-1 reverse transcriptase from Thai medicinal plants by in vitro assay. The promising medicinal plants, *E. scaber* was selected for purification and characterization its active compound. This is the first report on the *E. scaber* activity against HIV-1 reverse transcriptase.

MATERIALS AND METHODS

Medicinal plants

Thai medicinal plants were collected from the Siri-Rukkachad Medicinal Plant Garden, Faculty of Pharmacy, Mahidol University at Salaya Campus, Nakornpathom and Ubon Ratchathani Province (voucher specimens SR-MU-21 to SR-MU-43).

Plant extracts

Fresh plants (500 g) were chopped into small pieces and divided into two portions. One portion was blended with sterile distilled water at 4°C and another portion was macerated with 80% ethanol over night in the plant : solvent ratio of 1:2. The filtrates were filtered through cheesecloth and centrifuged at 3,500 g for 10 min. The supernatant was filtered through Whatmam No. 1 paper and sterile cellulose acetate membrane (pore size 0.45 µm), respectively. The water extract was lyophilized and the ethanol extract was evaporated at 50°C. The dried extracts were kept at 4oC. Each plant extract was resuspended in deionized distilled water (DDW) or 5 % dimethyl sulfoxide (DMSO) to obtain final concentration 4 µg/µl, for the preliminary test of RT inhibitory activity.

Tannin test

The presence of tannin in plant extracts can be detected by precipitation with gelatin in gelatin-salt block test (0.5% gelatin in 5% NaCl), and confirmed by coloration and formation of a precipitate assay with 10% ferric chloride[18,19].

HIV-1 RT inhibitory assay.

Recombinant HIV-1 reverse transcriptase was purchased from Calbiochem, USA. One unit is defined as the amount of enzyme that will incorporate 1.0 nmol of labeled dTTP into acid-insoluble material in 10 minutes at 37ºC, pH 8.3. Poly(rA)•oligo $(dT)_{12-18}$ and [methyl-^{3}H] Thymidine-5'-triphosphate (dTTP) (48 Ci/mmol) were purchased from Amersham Pharmacia Biotech, USA.

HIV-1 reverse transcriptase inhibitory assay was performed by the radiometric method[16]. A reaction mixture (100 µl) composed of 1M Tris-HCl (pH 8.0), 1M $Mg(OAc)_2$, 2 M KCl, 2 M dithiothreitol (DTT), 2.5, Nonidet P-40, deionized distilled water (DDW), bovine serum albumin, $(rA)_n$-$(dT)_{12-18}$ as template-primer, [methyl-^{3}H] dTTP as substrate, 1 Unit of HIV-1 reverse transcriptase, and 10 µl of the plant extract was dissolved in DDW or DMSO (complete

system + compound). The mixture was incubated at 37 °C for 1 hr. Two sets of negative control reactions were done under the same conditions: (i) without the plant extracts (complete system + RT), and (ii) the mixture consisting of solvent but the reverse transcriptase was omitted (complete system – RT). Doxorubicin was used instead of plant extract as the positive control.

The reaction was terminated by heat at 100 °C for 10 min, then spotted on Whatman DEAE-cellulose (DE-81) paper disc. The disc was dried and washed with 3% cold Na_2HPO_4, DDW and absolute ethanol (10 ml, each), respectively. The paper disc was dried at room temperature and immersed in the vial containing 5 ml scintillation fluid. The amount of the polymer product formed was determined by counting radioactivity with liquid scintillation analyzer (TRI-CARB 2000 TR, Packard Instrument Company, USA). Each sample was performed in triplicate. The inhibition ratio (%IR) of the tested plant extracts was calculated by the inhibition of incorporation of ^{3}H-dTTP into the newly synthesized DNA strand on the presence of plant extracts as follows:

$$(\%)\ IR = \frac{[\ 1-\ CPM\ (complete\ system + compound) - CPM\ (complete\ system - RT)]\ x100}{CPM\ (complete\ system + RT) - CPM(complete\ system - RT)}$$

The preliminary screening for HIV-1 reverse transcriptase inhibitor of plant extracts was performed at 200 µg/ml. The crude extracts which demonstrated more than 90% IR were selected for further testing.

Cytoxicity test

The protein was tested for toxicity toward brine shrimp (*Artemia salina* Leach) at concentrations of 10,100 and 1,000 µg/ml. Serial dilutions were made in six replicates in 96-well microplate, containing 200 µl of each concentration per well. The brine shrimp eggs were hatched in a shallow rectangular tray (22 x 23 cm) containing artificial seawater (3.8% sea salt) at room temperature. The eggs were sprinkled into the darken compartment and after the eggs hatched into free-swimming forms, the nauplii moved to the light side. After 48 hr the phototrophic nauplii were collected. A suspension of 6 nauplii (50 µl) was added to each well and the covered microplate was incubated for 24 hr at room temperature. The number of dead nauplii in each well was counted using a binocular microscope. LD50 was calculated by Reed-Muench method[20].

Purification of protein

Fresh *E. scaber* (1 kg) was sliced and blended with sterile distilled water at 4 °C, followed by centrifugation at 10,000 g (Centrikon T124, Kontron Instruments, Italy) for 30 min and filtered through Whatman filter paper No.1. The cleared supernatant was fractionated by precipitation with 30%, 60% and 80% ammonium sulfate saturation, respectively. The precipitate was collected by centrifugation (Centrikon T124, Kontron Instruments, Italy) at 10,000 g, 4°C for 30 min. The protein fractions were dialyzed (dialysis membrane MWCO 600 Da, Spectrum Medical, USA) against four changes of 2 liters of 50 mM sodium phosphate buffer pH 7.4 containing 1 mM EDTA at 4 °C. The dialysate were concentrated by the ultrafree centrifugal filter device with an exclusion limit at MWCO 10,000 Da (Millpore, USA). The concentrated samples were used for further purification.

SDS-PAGE was performed as described by Laemmli[21] using Protean® II xi Cell apparatus (Bio-Rad, USA). The slab gel (16 x 20 cm x 1 mm) containing 15% acrylamide for separating gel and 4% acrylamide for stacking gel was prepared. Samples were boiled with 2x sample buffer [10% SDS, 40% glycerol, 0.05% bromphenol blue,

10% of 2-mercaptoethanol in 1 M Tris-HCl buffer (pH 8.8)] for 5 min prior to loading. SDS-PAGE was performed with an electrode buffer (pH 8.3) (25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS) at 110 volts until the tracking dye reached the bottom of gel. Two reference lanes of the gel were cut, stained and destained to visualize the protein bands for locating the protein bands of interest in unstained gel. Then, the proteins and negative control (empty gel area) were cut and eluted with Tris-glycine buffer (0.3% Tris-base, 1.44% glycine, dissolved in DDW) using Electro-Eluter (Bio-Rad, USA) at 10 mA per sample for 3 hr. The eluted proteins were dialyzed against 50 mM phosphate buffer pH 7.4 containing 1 mM EDTA and concentrated by the ultrafree centrifugal filter device (Millpore, USA), then assayed for reverse transcriptase inhibitory activity.

Determination of protein concentration

Protein concentration was determined with the Bio-Rad protein assay dye reagent following the method described by Bradford[22]. The standard curve was obtained by plotting known concentrations of bovine serum albumin (0.2-1 mg/ml) versus A_{595} of the corresponding concentrations.

Determination of molecular weight of protein

The molecular weight of protein was determined by using SDS-PAGE. It was performed as described by Laemmli[21], using Mini Protean II apparatus (Bio-Rad, USA). The slab gel (6 x 8 cm x 0.75 mm) containing 15% acrylamide for separating gel and 4% acrylamide for stacking gel was prepared. The molecular weight markers (Bio-Rad, USA) containing phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) were used. Samples were treated as described previously prior to load. Electrophoresis was performed at 110 volts until the tracking dye reached the bottom of the gel. The protein bands in the gels were stained with Coomassie brilliant blue (0.1% Coomassie brilliant blue R250, 40% methanol, 10% glacial acetic acid), and destained with destaining solution (15% Methanol, 7 % glacial acetic acid).

The molecular weight of active proteins were estimated from the standard curve generated by plotting between the logarithmic of molecular weight of the broad range standard markers versus their relative mobility (R_f).

Isoelectric focusing (IEF)

Isoelectric focusing was performed with optimized method for IEF with PhastGel IEF 3-9 using Phast System (Amersham Pharmacia Biotech, USA). A PhastGel IEF media (IEF 3-9) was run in the Phast System separation unit and subsequently stained in the Phastsystem development unit with Pharmacia Coomassie staining method as described by the manufacturer. Bio-Rad' s IEF standards, a mixture of fifteen natural proteins with isoelectric points (pIs) ranging from 3.6 to 6.6, and 4.45 to 9.6 were used.

Amino acid sequencing

1. Protein Immobilization

After SDS-PAGE, the gel was subjected to electroblotting using Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad) by the method described by Applied Biosystems company. The immobilization membrane (ProBlott™) was used to transfer the protein band. The membrane was fixed with 100% (v/v) methanol for 10 sec, then placed in electroblotting buffer. The gel was removed from the electrophoresis cell and soaked in electroblotting buffer for 5 min. The gel was then sandwiched between pre-wet membrane, three layers of Whatman No. 1 paper, and sponges. The electroblotting was performed with the same buffer under a constant volt of 50 volts at room temperature for 30 min. The membrane was then removed from the transblotting sandwich and rinsed with deionized distilled water prior to staining. The membrane was saturated with 100% (v/v) methanol for 10 min and stained with 0.1% (v/v) Coomassie brilliant blue R-250

in 40% (v/v) methanol and 1% (v/v) glacial acetic acid for 1 min at room temperature. The membrane was then destained with 50% (v/v) methanol until protein bands appeared and rinsed extensively with deionized distilled water. The bands were excised and stored at –20 °C until sequencing.

2. N-terminus amino acid sequencing

Amino acid sequencing was performed by method described by Applied Biosystem Company. Automated cycles of Edman degradation were performed by gas phase protein sequencer (Applied Biosystem model 476A) and phenylthiohydantoin (PTH) amino acid derivatives were automatically identified by data analysis apparatus (Applied Biosystems model 610A). The membrane sample from electroblotting was dried by a SC100A Speedvac Plus (Savant) and placed into the Blott cartridge. A zitex seal was placed between the two block and the cartridge placed in the cartridge holder. The resulting PTH amino acids were analyzed on an Applied Biosystems PTH-C18 reversed phase cartridge column (220 x 2.1 mm). All chemicals used in Sequencing and PTH amino acid analysis were obtained from Applied Biosystems (Foster City, USA).

RESULT

The % inhibitory of 200 µg/ml of 22 plants extracted to HIV-1 RT and tannins test were shown in Table 1. Therefore the water extract of *E. scaber* (no tannin) was

Table 1. HIV-1 inhibitory effect of Thai medicinal plants and the results of tannin test.

Family/Botanical name	Local name	Part used (w/w)	%Yield	Tannins	% Inhibition Ratio	
					EtOH	H2O
Annonaceae						
Annona reticulata L.	Noinong	Stem barks	ND	-	0	20
Annona muricata L.	Thurian thet	Stem barks	ND	-	0	30
Polyalthia suberosa (Roxb.) Thwaites	Klueng Klom	Stem barks	1.5	-ve	0	0
Polyalthia cerasoides	Ka-Chain	Stem barks	ND	+ve	9.67	12.5
(Roxb.) Beddome	Root	ND	+ve	9.63	22.5	
Polyalthia debilis	Kluai Tao	Root	ND	-ve	ND	12.97
(Pierre) Finet et Gagnep.						
Polyalthia evecta						
(Pierre) Finet et Gagnep.	Tap Tao	Root	1.8	-ve	0	32
Mitrephora vandaeflora Kurz	Mapuan	Stem barks	1.5	-ve	0	28.5
Ellipeiopsis ferruginea	Teen Tang Tia	Root	ND	-ve	3.8	18.4
(Buchanan-Hamilton ex J.D. Hook. et Thomsom) R.E. Fries						
Goniothalamus laoticus	Khaolam	Leaves	8.1	-ve	0	42.5
(Finet et Gagnep.) Tien Ban Bot.						
Euphorbiaceae						
Jatropha curcas L.	Sabu-dam	Stem barks	0.66	+ve	0	97.5
Compositae						
Elephantopus scaber L.	Do-mai-ru- lom	Leaves+Root	1.24	+ve	26.44	96.93
Chrysanthemum indicum L.	Kek-huai	Flowers	0.57	+ve	0	37.0
Cucurbitaceae						
Momordica charantia L. (protein 120 µg/ml)	Mara	Seeds	ND	ND	ND	88.75
Euphorbiaceae						

Table 1. HIV-1 inhibitory effect of Thai medicinal plants and the results of tannin test. (Continued)

Family/Botanical name	Local name	Part used (w/w)	%Yield	Tannins	% Inhibition Ratio	
					EtOH	H2O
Securinega virosa Baill.	Kang pla khao	Branches	1.0	+ve	70.2	1.45
		Branches+ Leaves	0.57	+ve	88.25	56.67
Euphorbiaceae						
Securinega leucopyrus Muell. Arg.	Kang pla daeng	Leaves	4.93	-	26.8	0
	Branches	0.61	-	30.3	0	
Guttiferae						
Garcinia fusca Pierre	Madan-pa	Fruits	1.77	+	48.9	12.3
Garcinia cowa Roxb.	Cha-muang	Fruits	1.92	-	27.5	0
Garcinia cataractaris Whitmore	Ung-pao	Fruits	1.05	-	19.2	0
Calophyllum inophyllum L.	Kra-thing	Branches + Leaves	0.55	-	0	0
		Fruit	0.4	-	0	10.89
Amaryllidaceae Hypoxidoide						
Curculigo orchidoides Gaertn.	Wan-phrao	Rhizomes	0.37	+	10.87	13.89
Simaroubaceae						
Harrisonia perforata Merr.	Seefan khontha	Leaves	4.48	+	49.53	0
Sonneratiaceae						
Sonneratia caseolaris Engler	Lum-phu	Branches	1.3	+	ND	37.38

Remarks - - = negative
+ = positive
ND = not determine

selected for determination of IC_{50}. The results showed that the IC_{50} of leaf and root was 69.9 and 107.5 µg/ml, respectivly. Lineweaver-Burk Plots (Figure 1) determined mode of action. It was kinetically analysed by changing the concentration of the triphosphate substrate in the presence of various concentrations of the inhibitory substances under the conditions described in Materials and Methods. Result found that the mode of action was non-competitive inhibition with respect to the substrate (dTTP). Moreover, they were extremely low toxic to brine shrimp (LD_{50} > 1000 µg/ml).

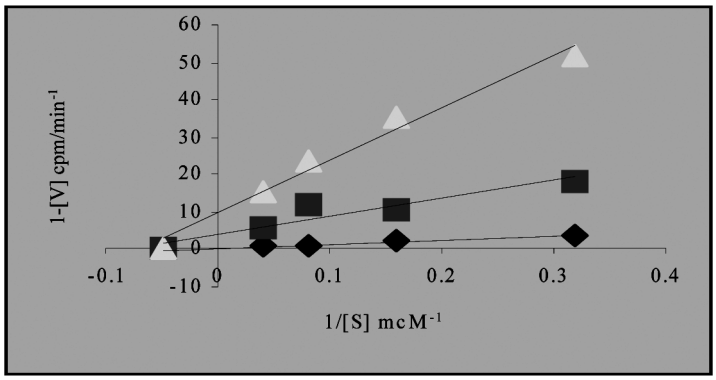

Figure 1. Lineweaver-Burk plot for the inhibition of HIV-1 RT by water extract of *E. scaber* in the presence of various concentrations of dTTP. Concentrations of protein were 0 ,70 and 140 µg/ml.

Crude water extracts from leaves and root were precipitated with 30, 30-60, and 60-80% ammonium persulfate, respectively. Proteins were separated by SDS-PAGE, the protein patterns were shown in Figure 2.

Protein patterns were similar between root and leaves. The precipitate protein from 60-80% (leaves) and 30-60% (root) were selected for separation by 12% SDS-PAGE, using Mini Protein II apparatus (Bio-Rad) (Figure 3). Each band of protein was eluted from SDS-PAGE and test for anti HIV-RT.

The IC50 of purified protein was 4.29 µg/ml. The molecular weight was approximately 34.5 kDa (Figure 3, lane 3), this protein was designated as Elep-34. It's isoelectric point (pI) was 4.65 and ten amino acid residues from N-terminus were found to be Met-Ala-Ala-Ala-Glu-Pro-Phe-Gly-Asp-Leu- Asn- Leu (Table 2).

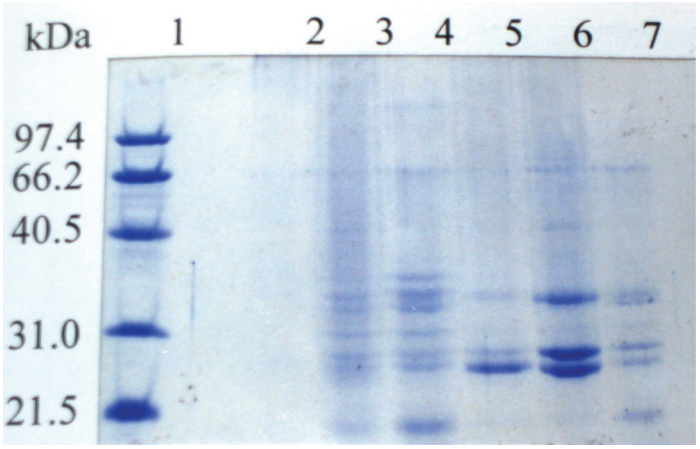

Figure 2. SDS-PAGE patterns of protein from *E. scaber* L. *Lane 1*: Protein molecular weight maker, *lane 2-4*: 30, 30-60, 60-80% protein from leaves and *lane 5-7*: 30, 30-60, 60-80% protein from root.

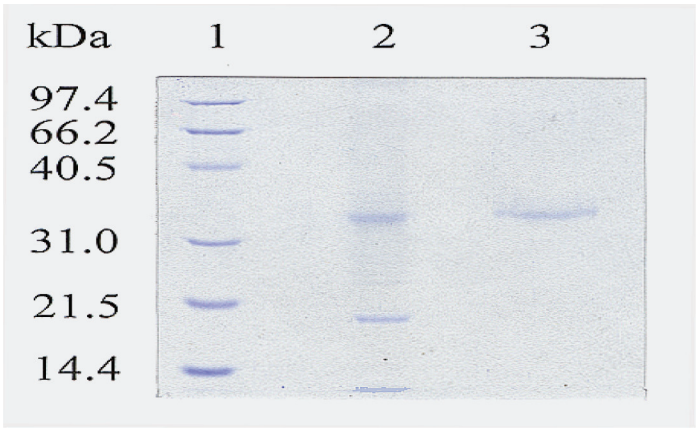

Figure 3. SDS-PAGE patterns of protein of *E. scaber* L. *lane 1*: Protein molecular weight marker, *lane 2*: 60-80% from leaves and *lane 3*: Elep-34 protein

Table 2. Comparison of amino acid sequence at N-terminal of *E. scaber* and other HIV–1 reverse transcriptase inhibitor from plants

Plant	N-terminal	Reference
E. scaber	Met Ala Ala Ala Gln Pro Phe Gly Asn Leu	This study
MRK29	Asp Val Asn Phe Arg Leu Ser Gly Ala Asp	Jiratchriyakul *et al*. 2001
MAP 30 *(M. charantia)*	Met Val Lys Cys Leu Leu Leu Ser Phe Leu	GenBank No. AAB35194
MAP 30 chain A *(M. charantia)*	Asp Val Asn Phe Asp Leu Ser Thr Ala Thr	GenBank No. 1D8VA
Momordin a *(M. charantia)*	Asp Val Ser Phe Arg Leu Ser Gly Ala Asp	GenBank No. 1918208A
Momordin II *(M. charantia)*	M et Val Lys Cys Leu Leu Leu Ser Phe Leu	GenBank No. CAA78166

DISCUSSION

The twenty-two medicinal plants showed that the water extracts of *E. scaber* (leaves and roots), *J. curcas* (leaves and barks), and the 80% ethanol extract of *S. virosa* (branches and leaves) exhibited the high inhibitory effect on HIV-1 RT with 96.93, 97.25, and 88.25% IR, respectively. The plant extracts that possessed tannins were excluded because tannins could be the non-specific inhibitory activity to HIV-1 RT. Tannins are a group of compounds occurring naturally in many plants, they have been studied as RT inhibitors. Kakiuchi *et al*.[23] reported that dimeric ellagitannins and galloylate condensed-tannins were thought to inhibit RT by attaching to the grooves of the double-stranded chains, inhibiting the formation of a template-primer-enzyme-nucleotide complex and more sensitive when poly(rA)-oligo(dT) was template-primer than poly(rC)-oligo(dG)[23]. Some research works removed tannin before test for RT-inhibitor[19]. In this study, *J. curcas* (leaves and barks) exihited the most potent activity but it tannin test was positive. Therefore, water extract of *E. scaber* was selected for further study, because there is no tannins and its anti-HIV-1 RT activity was strong and stronger than 80% ethanol extract. The result was similar to Kusumoto *et al*.[24]. They found that the water extract from leaves of *E. scaber* exhibited the inhibitory effect higher than 70% ethanol. Its IC_{50} was 10 µg/ml by using the poly(rC)-oligo(dG) and AMV-reverse transcriptase. The result indicated that RT assay was dependent on many factors in reaction, type of enzyme (AMV-RT, HIV-RT, and MuLV-RT) and type of template-primer (poly(rA)-oligo(dT) or poly(rC)-oligo (dG)), the inhibitory more stronger in poly (rA)-oligo(dT) than poly(rC)-oligo(dG) in most inhibitor (Kusumoto, 1992). The mode of action was non-competitive with respect to the substance (dTTP) non-competitive with respect to the substance (dTTP)

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