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Original Article

Inhibition of HIV-1 Reverse Transcriptase of Selected Indonesia Medicinal Plants and Isolation of the Inhibitor from *Erythrina variegata* L. Leaves

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ABSTRACT

Objective: This research was conducted to screen inhibition of HIV-1 reverse transcriptase activity of selected Indonesia medicinal plants and to isolate HIV-1 reverse transcriptase inhibitor from *Erythrina variegata* leaves. **Method:** Screening inhibition of HIV-1 RT activity of selected Indonesia medicinal plants and isolated compounds were performed using HIV-1 RT colorimetric assay. The isolation of HIV-1 reverse transcriptase inhibitor was conducted using chromatography technique. The isolated compound was determined based on the data of UV, IR spectrophotometry, MS, 1D and 2D NMR spectroscopy. **Results:** Beside that, *in vitro* study of the leaves methanolic extract exhibited inhibition against HIV-1 RT activity with percent inhibition of 97.64% at concentration of 5 mg/mL. Ethyl acetate fraction from the extract showed the strongest HIV-1 RT inbitory activity with IC_{50} of 429.28 µg/mL. Isolation the HIV-1 RT inhibitor from the fraction give compound 1. **Conclusion:** *Erythrina variegata* leave extract exhibited potent inhibition on HIV-1 RT activity. The isolated compound from the leaves was determined as apigenin-7-*O*-β-D-glucopyranoside and demonstrated HIV-1 RT inhibitory activity with the IC_{50} value of 100.59 µg/mL.

Key words: *Erythrina variegata*, Flavonoid, HIV-1 RT, *in vitro*.

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INTRODUCTION

Human immunodeficiency virus (HIV) is the virus that can lead to HIV infection. During HIV infection, the virus attacks and destroys CD4 cells that cause a disruption in the immune system in the body to fight infection. Acquired immunodeficiency syndrome (AIDS) is the final stage of HIV infection that causes decreased immunity.[1] Based on the report from UNAIDS, globally about 34 million people have been infected of HIV by 2011. It is estimated that 0.8% of people worldwide were aged 15–49 years HIV infected. Asia has a number of nearly 5 million people living with HIV are spread in South Asia, Southeast Asia and East Asia.[2] HIV-1 reverse transcriptase (RT) is a DNA-dependent polymerase that served as the catalyst of the synthesis of double-stranded DNA copies of HIV-RNA. This enzyme is an essential component of the life cycle of HIV-1 and as a target for anti-HIV therapy. drug therapy for HIV infection currently include inhibitors of RT and protease enzymes.[3]

Natural products, especially derived from plants, have been known as a source of anti-HIV drugs. Some of them showed inhibitory activity against a variety of processes in HIV-1 infection.[4] Some plants have been known to benefit as an anti-HIV-1 from different countries. Syahdi *et al.* (2012) have performed virtual screening of HIV-1 transcriptase inhibitor base on Indonesian herbal database.[5] The study resulted in top ten compounds which have the prospect to be developed as HIV-1 reverse transcriptase inhibitor. Based on that study, we selected ten plants which contained the compounds and evaluated their inhibitory activity *in vitro* against HIV-1 reverse transcriptase. The extract demonstrated the strongest HIV-1 RT inhibitory activity was further fractionated for isolation of the HIV-1 RT inhibitor.

METHODS

Plant collection

Plants were collected from Ministry of Health Garden in Citereup, West Java, Indonesia, and were authenticated by Indonesia Institute of Sciences, Cibinong, West Java, Indonesia. The voucher specimens were deposited in Herbarium and Pharmacognosy Laboratory, Universitas Indonesia.

Extracts preparation

Samples powder were macerated using 50% methanol, then filtered. The same procedure was repeated two times. Organic solvents were combined, and the evaporated under pressures at 50°C to give extracts. The methanolic extracts were dissolved using 10% DMSO to generate sample solution for assay at 5 mg/mL. Lamivudine was used as a standard assay due to lamivudine is antiretroviral from NRTI class. Lamivudine was dissolved using 10% DMSO solvent to obtain standard concentration of lamivudine at 200 ppm.

Extraction and Partition

The dried leaves powder of *Erythrina variegata* was macerated using methanol. Methanolic extracts were evaporated in a rotary vacuum evaporator at 50°C. The extract was dispersed in hot water and then partitioned with n-hexane, ethyl acetate, subsequently. Organic layers were evaporated under pressure to give hexane and ethyl acetate extracts. The aqueous layer was dissolved in methanol. The dissolved methanol was evaporated to give methanol fraction.

Isolation

Isolation was conducted using column chromatography and preparative thin layer chromatography (TLC). Ethyl acetate fraction was fractionated

by column chromatography (3×50 cm) using silica gel 60 as a stationary phase, a mobile phase of n-hexane and ethyl acetate in gradient polarity system. The fractions were chromatographed using preparative TLC, and further purification was conducted by recrystallization. The isolated compound was identified to determine the chemical structure. Elucidation of the structure was performed by analyzing the spectral data of UV-Vis, MS, IR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, HMQC, and HMBC.

HIV-1 Reverse Transcriptase (RT) Inhibitory Assay

HIV-1 reverse transcriptase (RT) inhibitory assay was designed to be used in research studies as a method for the quantitative determination of RT activity in cell culture and other biological samples. This assay was used to determine the spread of retroviruses in mammalian cell culture infected. Reverse Transcriptase colorimetric kit assay was obtained from Roche, Germany. This assay was also used as a method of *in vitro* screening for RT inhibitor compounds. In this assay, lamivudine was used as positive control.[6] The first step, HIV-1 Reverse Transcriptase (HIV-1 RT) enzyme recombinant (0.2 ng/ µL) were placed to a microplate. The inhibitor dissolved in lysis buffer and the template were added into a microplate containing (HIV-1 RT) enzyme. Lysis buffer without the enzyme used as a blank control. Then a microplate incubated for 1 h at 37°C. The solution cleaned and washed with washing buffer five times at a temperature of 15–25°C. Washing buffer then removed carefully. After the microplate clean, anti-DIG-POD (200 U/mL) was added on each microplate and then covered with plastic back cover and incubated for 1 h at 37°C. Once clean, ABTS substrate solution was added into each microplate and incubated for 10–30 minutes with a rotation of 250 rpm at a temperature of 15–25°C until the color changes to green. Measurements carried out on a sample absorbance at λ 405 nm.

RESULTS

Table 1 shows HIV-1 RT inhibition activity of the extracts from selected medicinal plants. There are five plants that have inhibitory activity with a value of more than 90% inhibition including *Erythrina variegata*, *Garcinia mangostana*, *Morus alba*, *Psidium guajava* and *Pluchea indica*. *Erythrina variegata* has an inhibitory activity of 97.64 %. Inhibitory activity of HIV-1 RT by *Erythrina variegata* made possible by the erycristagallin content.[6] *Garcinia mangostana* has a high activity in the inhibition of HIV-1 RT that is 98.01%.

Table 2 shows the yield of each fraction from *E. variegata* leaves extract. Ethyl acetate fraction was further fractionated and gave precipitate. After purification, the isolate then separate using thin layer chromatography with ethyl acetate:n-hexane:chloroform (4:1:1) as eluent. Isolated compound was a yellowish-white powder. The spot of the isolated compound on the plate chromatography showed bright yellow spot under UV light when sprayed with the $AlCl_3$ color developer reagent. This is specific for flavonoid. UV_{max}: 275 and 355 nm. MS spectra: m/z 432.1840 [M^+]. FT-IR υ_{max} (cm^{-1}): 3237, 2930, 1741, 1178 and 819 cm^{-1}. Table 3 shows data of NMR spectra of the isolated compound.

Reverse transcriptase inhibitory activity carried out by using a kit of HIV-1 RT enzyme (Roche, Germany) using lamivudine as a positive control. HIV-1 RT Inhibitory activity assay was preceded by testing of *Erythrina variegata* isolate with lamivudine as standard at concentration range of 500 ppm, 400 ppm, 300 ppm, 200 ppm, 100 ppm.

Figure 2A shows a value of 50% inhibitory concentration (IC_{50}) of lamivudine was 128.86 µg/mL. According to data from lamivudine (Epivir®) assay, the lamivudine IC_{50} value of *in vitro* testing is in the range of 2 nM to 15 µM. The Figure 2B shows IC_{50} inhibition of HIV-1 RT enzyme by the isolate is 100.59 µg/mL. From the IC_{50} value, it is known that isolate has higher activity compared to lamivudine as positive control.

Table 1: HIV-1 RT inhibition ratio of each extract

Samples (5 mg/mL)	Part of plants	Inhibition ratio (%)
Morus alba	Leaves	98.45
Garcinia mangostana	Leaves	98.01
Erythrina variegata	Leaves	97.64
Psidium guajava	Leaves	97.21
Plucea indica	Leaves	94.37
Vitex trifolia	Leaves	86.98
Foeniculum vulgare	Seeds	84.73
Erythrina cristagalli	Leaves	76.65
Theobroma cacao	Seeds	69.57
Strychnos lucida	Stems	51.88
Cinchona ledgeriana	Barks	47.76
Tinospora rumpii	Stems	31.12
Mangifera indica	Leaves	26.64
Artocarpus heterophyllus	Stems	24.83
Hibiscus mutabilis	Leaves	11.09
Lamivudine 200 ppm		98.45

Table 2: Partitioned fractions of *E. variegata* leaves extract

Fractions	Weight (g)
n-hexane	142
Ethyl acetate	36
Methanol	90

DISCUSSION

Some studies reported natural products inhibited HIV-1 RT activity. *Garcinia mangostana* inhibit the enzyme by the content of α-mangostin, β-mangostin and γ-mangostin. According to the research conducted by Chin and Kinghorn 2008, β-mangostin showed inhibitory activity against HIV-1 RT of 42 %.[7] *Morus alba* has an inhibitory activity of 98.45 % by the morusin content. *Psidium guajava* has the inhibitory activity of 97.21 % associated with the content of quercetin and guajiverin. While *Pluchea indica* has an inhibitory activity of 94.37 % due to plucheoside A content.[6]

Erythrina variegata L. (Fabaceae) grows in tropical regions such as Indonesia. The leaves were used as traditional medicine to treat pathogenic parasites and reduces the joint pain in India, China, and Southeast Asia. Erycristagallin isolated from the leaves showed antimicrobial activity against methicillin-resistant *Staphylococcus aureus*. The methanolic extract of leaves showed significant antinociceptive activity on writhing response in acetic acid induced.[5,6] There is no report about the anti-HIV-1 RT activity of *E. variegata*.

The structure of isolated compound from *E. variegata* leaves was elucidated based on spectral data. The isolated compound was suggested as flavonoid due to the TLC chromatogram after was sprayed with $AlCl_3$ solution. $AlCl_3$ formed complex with hydroxyl groups and neighboring ketone (C-4 in ring C and C-5 on ring A).[7] This result was supported by UV-Vis spectrum.[8] The UV spectrum formed two bands on λ 355 and 275 nm which can be deduced that the isolate was flavonoid. The UV spectrum obtained was similar to the structure of flavones.[9] The MS spectra showed the peak ion fragment at m/z 432.1840 [M^+] which

Table 3: ^{13}C-NMR, ^{1}H-NMR, HSQC dan HMBC of isolate (DMSO-*d6*, 500 MHz)

Position	δ*C* ppm	δ*H* ppm	HSQC	HMBC
2	121.0, *s*	-	-C-	-
3	99.6, *s*	6.47, *s*	-CH-	-
4	180.5, *s*	-	-C-	-
9	106.1, *s*	-	-C-	-
5	163.0, *s*	-	-C-	-
6	94.5, *d*	6.71 (*d*, *J* = 2 Hz)	-CH-	-
7	161.6, *s*	-	-C-	-
8	94.5, *d*	6.72 (*d*, *J* = 2 Hz)	-CH-	C-9, C-10, C-5
10	157.2, *s*	-	-C-	-
1'	122.6, *s*	-	-C-	-
2'	130.2, *d*	7.39 (*d*, *J* = 8.5 Hz)	-CH-	C-2', C-2
3'	115.1, *d*	6.82 (*d*, *J* = 8.5 Hz)	-CH-	C-1', C-3'
4'	154.6, *s*	-	-C-	-
5'	115.1, *d*	6,88 (*d*, *J* = 8.5 Hz)	-CH-	C-1', C-3'
6'	130.2, *d*	7.40 (*d*, *J* = 8.5 Hz)	-CH-	C-2', C-2
1"	99.9, *d*	5.06 (*d*, *J* = 7.0 Hz)	-CH-	C-7
2"	77.2, *d*	3.451	-CH-	-
3"	73.1, *d*	3.263	-CH-	-
4"	69.6, *d*	3.168	-CH-	-
5"	76.4, *d*	3.278	-CH-	-
6"	67.4, *t*	4.10 (*dd*, *J* = 8.5 and 5 Hz)	-CH_2-	-
OH-5	-	12.93	-	-
OH-4'	-	9.62	-	-

showed the molecular formula $C_{21}H_{20}O_{10}$. Fourier Transform Infrared (FT-IR) spectra showed the wave number υ = 3237 cm^{-1} indicated the presence of OH groups or aromatic carboxylic; υ = 2930 cm^{-1} indicated the presence of alkane group, on υ = 1741 cm^{-1} indicated ketone group (C=O), in υ = 1178 cm^{-1} indicated the presence of carboxylic groups or ester (COOH or COOR) and the υ = 819 cm^{-1} indicated the presence of double bond or alkene group (R2C = CHR).

The elucidation of the structure of the isolated compound was further confirmed by NMR spectra consisting of ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC. ^{1}H-NMR spectrum gives the information about the number of protons, chemical and electronic environment of protons.[3] ^{1}H-NMR, ^{13}C-NMR and 2D NMR spectra confirmed that the compound was flavonoid-glucoside. Aromatic ring showed at δ 6.71 (*J* = 2 Hz, H-6) and δ 6.72 (*J* = 2 Hz, H-8). The two singlets at δ 12.9 and 9.62 indicated two hydroxy groups and were located at the position of C-4'(δ 154.6) and C-5 (δ 163.0), respectively. The position of the substituted groups were characterized by the presence of ^{1}H-^{13}C correlations in the HMBC spectrum. The HMBC spectrum shows a correlation between the anomeric proton at δ 5.06 with C-7 (δ 161.6) that indicated the position of the glucoside moiety. Based on the spectral data, the structure of the isolated compound was determined as apigenin-7-*O*-β-D-glucopiranoside (Figure 1).

Apigenin-7-*O*-β-D-glucopiranoside has known as apigetrin or cosmosiin. This compound was found in the *Cosmos bipinnatus* flower, *Zinnia elegans* and many other plants. Cosmosiin has an antiplatelet activity with IC_{50} of 0.18 mg/mL. Cosmosiin also showed the anti-amoeba activity with IC_{50} 22.3μg/mL.[10] The other study showed that several flavonoid compounds that capable to inhibit HIV-1 RT enzyme. Xanthohumol,

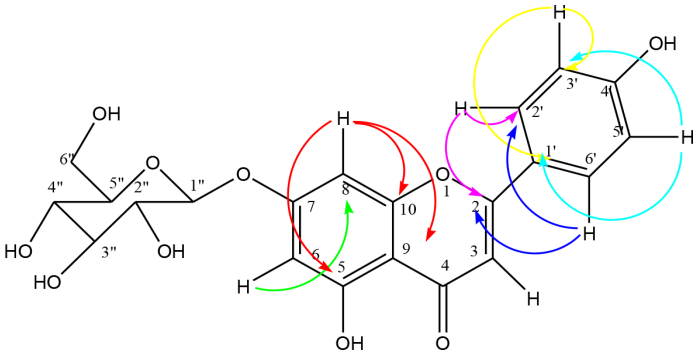

Figure 1: Chemical structure and HMBC correlation of isolate

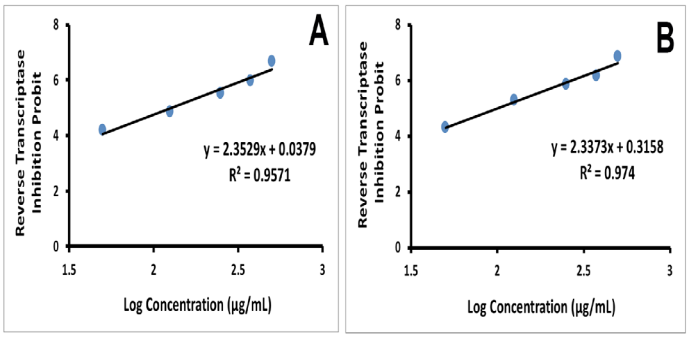

Figure 2: Reverse transcriptase inhibition curve by (A) lamivudine, and (B) isolate.

prenylchalcone from *Humulus lupulus* are able to inhibit the replication of HIV-1 in PBMCs with EC_{50} 20.74 mg/ mL.[11] *Vitex negundo* L. ethanolic extract has inhibitory activity against HIV-1 RT of 92.8% at a concentration of 200 µg/mL. The extract was analyzed using HPLC shown that it contains flavonoids such as rutin, luteolin, myricetin, quercetin, kaemferol, quercetagetin.[12]

In this study as standard was used lamivudine. Le *et al*. (2007) reported that lamivudine revealed HIV-1 RT inhibition activity with IC_{50} value of 6.5 µM.[13] The isolated compound is flavonoid and showed strong anti-HIV activity. The result of this study supported anti HIV activity of flavonoid.[14] Some phytochemicals such as tannins, gallotannins, ellagitannins, cyanidin, and flavonoids from *Terminalia catappa* were reported having anti-HIV activity.[15] Ethanolic leaves extract of *Vitex negundo* demonstrated good HIV-1 Reverse Transcriptase activity of 92.8% at 200 mg/ml. The extract contained flavonoid such as: kaemferol, myricetin, quercetin, quercetagetin, isorhamnetin and luteolin.[12] Another study reported that flavanone and flavonol glycosides isolated from the leaves of *Thevetia peruviana* exhibited HIV-1 reverse transcriptase and HIV-1 integrase inhibitory activities.[14] Flavonoid myricetin showed potential activity against HIV-1 *in vitro* microbicide activity model, and also showing insignificant cytotoxic effects.[16] Luteolin and its derivates from *Colues parvifolius* exhibited inhibitory activities against HIV-1 IN.[17]

CONCLUSION

This research showed that the *Erythrina variegata* leaves extract were potential as HIV-1 RT inhibitor. The isolated compound was determined as apigenin-7-*O*-β-D-glucopiranoside or also known as apigetrin or cosmosiin. The isolate was also shown to be active against HIV-1 RT enzyme with IC_{50} of 100.59 µg/mL.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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