

***In vitro* anti-HIV activity of ethanol extract from gandarusa (*Justicia gendarussa* Burm. f) leaves**

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Abstract

Anti retroviral drugs for HIV has problems with uncomfortable side effects and that endanger the lives of HIV sufferers. Several herbs have been empirically proven to have an effect on HIV eradication through inhibition of reverse transcriptase. One of such antiviral herbs is *Justicia gendarussa* (*J. gendarussa*). The aim of research is to evaluate anti-HIV activity of 70% fractionated-ethanol extract (with releasing alkaloids) and 70% ethanol extract (without releasing alkaloids) of *J. gendarussa* leaves on in vitro HIV-infected of MOLT-4 cells. The effect of the extracts in inhibiting viral replication and fusion process on acute HIV infection was identified through syncytia formation assay. Effect of the extracts on HIV p24 antigen was evaluated using HIV-1 p24 ELISA kit. It was found that 70% fractionated-ethanol extract and 70% ethanol extract of *J. gendarussa* leaves significantly inhibited of HIV replication by inhibition of syncytia formation, where the 50% effective concentration (EC50) values of the 70% fractionated-ethanol extract and 70% ethanol extract are 70.5 µg/mL and 228.7 µg/mL, respectively. Both of the extracts were also significantly inhibited HIV replication by decreasing HIV p24 antigen level where the EC 50 values of the 70% fractionated-ethanol extract and 70% ethanol extract are 88.8 µg/mL and 540.7 µg/mL, respectively. Moreover, it was found that 70% fractionated-ethanol extract of *J. gendarussa* leaves has anti-HIV activity since its EC50 values less than 100 µg/mL. It was concluded that *J. gendarussa* could be potentially developed into a phyto-pharmaceutical product due to its anti-HIV activity.

Introduction

Human Immunodeficiency Virus (HIV) is a retrovirus that infects human immune system, causing decline of human immune system and leading to a condition called Acquired Immune Deficiency Syndrome (AIDS).¹ HIV transmission causes high number of HIV infection and high number of death in the world. Antiretroviral (ARV) is a therapy that used to treat patients with HIV infection. ARV has some disadvantages, namely toxicity associated with inability of patients to withstand adverse effects of drugs, high cost of ARV therapy, limited availability of drugs combination,² lack of any curative effect and viral resistance to ARV drugs.³⁻⁵

One alternative strategy for prevention of HIV infection is using medicinal plants. Some medical plants have potential sources to discovery of new active agent with anti-HIV activity. Medicinal plants are relatively non-toxic. Hence, medicinal plants more tolerable than chemical drugs.⁶ *Justicia gendarussa* Burm.f. has been known to have anti-HIV activity⁷ mainly from the inhibition of reverse transcriptase enzym. At 200 µg/mL concentrations, water extracts of *J. gendarussa* (aerial part) has HIV-1 RT inhibition ratio (% IR) higher than 90%.⁸ Leaves of *J. gendarussa* plants contain a substituted aromatic amine,⁹ flavonoid glycosides including gendarusin A and B,¹⁰ and justidrusamide alkaloids A, B, C, and D.¹¹ Gendarusin A is a major compound of *J. gendarussa* leaves that has anti-HIV activity on plasma blood of HIV patients in vitro with IC₅₀ value was 10.24 µg/mL. A previous study found that an alkaloids-free 70% ethanol extract of *J. gendarussa* leaves could significantly reduce viral load in blood plasma of HIV patients. Viral load reduction of an alkaloids-free 70% ethanol extract of *J. gendarussa* leaves greater than the viral load reduction of methanol extract.¹² In addition, 70% alkaloids-free ethanol extract of *J. gendarussa* leaves has inhibitory activity of the reverse transcriptase enzyme.¹³

In this study, the effect of 70 % fractionated-ethanol extract (with releasing alkaloids) and 70% ethanol extract (without releasing alkaloids) of *J. gendarussa* leaves on in vitro HIV-infected of MOLT-4 cell were compared using syncytia formation and p24 antigen assay.

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Materials and Methods

Preparation of the extract

Plant Material

Leaves of *Justicia gendarussa* used in this study were obtained from a cultivated crop in Trawas, Mojokerto, East Java province, Indonesia. The medicinal plants were identified by Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya.

The ethanol extracts

Powder of *J. gendarussa* leaves was divided into two fractions, namely releasing-alkaloid leaf powder and a non releasing-alkaloid leaf powder. Releasing-alkaloid leaf powder was prepared by adding an acid solution so that the alkaloids were changed into water-soluble salt. Both

of powders were extracted by using 70% ethanol during 3'24 hours in a macerator to obtain filtrate. Then, the filtrate then was evaporated using a rotary evaporator (Buchi). After that, the extracts were dried at 50 °C until 70% fractionated-ethanol extract (alkaloid-free; 2.4% w/w) and 70% ethanol extract (10.8% w/w) could be obtained.

Detection of Alkaloids in *J. gendarussa* Leaves Extracts

Content of alkaloids in extracts of *J. gendarussa* leaves was analyzed by using Thin Layer Chromatography (TLC) methods. We used silica gel GF₂₅₄ TLC plate (Merck) as stationary phase and dichloromethane:methanol (9:1) as mobile phase. The Dragendorff reagent was used as spray reagent to qualitatively identify the presence of alkaloids compounds. The presence of alkaloids compounds was marked with orange spot. Extract of *Piper nigrum* fruits was used as a comparator of the presence of alkaloids in the *J. gendarussa* extracts.

Detection of gendarusin A in *J. gendarussa* Leaves Extracts

The content of gendarusin A, the major flavonoid in *J. gendarussa* leaves, was analyzed by High Performance Liquid Chromatography (HPLC) methods using HPLC Agilent 1100, reverse phase NovaPack® column C-18 3.9'150 mm (Waters) as stationary phase and mixture of water and methanol (water:methanol =30:70) as mobile phase. The analysis was performed at these conditions: flow rate 1 mL/min, stop time 15 minutes, and UV detector at 254 nm wavelength.

Anti-HIV assay

Preparation of ethanol extracts stock solutions

A stock solution was made by dissolve 100.0 mg of each extract in a 1000 µL DMSO. Then, the solution was diluted by using RPMI-1640 medium (Gibco) with 10% Fetal Bovine Serum (FBS) (Gibco) (inactivated at 56 °C for 30 min) to obtain various concentrations (7.8; 15.6; 31.2; 62.5; 125.0; 250.0; 500.0; and 1000.0 mg/ml) for each trial extract. Concentration of DMSO used in this research less than 1 %. At this concentration level, DMSO does not affect viability.¹⁴

Preparation of cell and virus

MOLT-4 cells clone#8 (human T lymphocytes cancer cells line)¹⁵ were obtained from Bio-safety Level-3 facility CRC-ERID, Institute of Tropical Disease, Surabaya. MOLT-4 cells were cultured on a RPMI-1640 media, with 10%FBS and were

kept in CCF T₂₅ at 37°C in a 5% CO₂ incubator (Sanyo).

HIV virus isolate from a seropositive HIV donor were obtained from Bio-safety Level-3 facility CRC-ERID, Institute of Tropical Disease, Surabaya. The HIV virus isolate was labeled that labeled by IDU-18 code. HIV isolate were cultured on MT-4 cell in RPMI-1640 medium, with 10% FBS and kept in CCF T₂₅ at 37 °C in 5% CO₂ incubator (Sanyo).

Syncytia Formation Assay

Effect of *J. gendarussa* leaves extracts on HIV-infected MOLT-4 cell was measured by using syncytia formation. A 100 mL of the extract solution with various concentrations were plated in each well on 96-well micro plate. The treatment group and negative control group (without extract) were tested in triple replication. 50 mL of 2'10⁴ cell/mL MT-4/HIV were added to each well and were incubated for 30 minutes at 37°C in a 5% CO₂ incubator. After 30 minute of incubation, 50 mL of 4'10⁵ cell/mL MOLT-4 cells were added into each well. The total volume in each well was 200 µL. The microplate was incubated for 72 hours at 37°C in a 5% CO₂ incubator. After 72 hours of post-infection, the syncytia formation (giant cell with ≥4 nucleus per cell)¹⁶ in

each well of microplate was observed and calculated under an inverted microscope (Olympus) (10'10).¹⁷ The inhibitory percentage of syncytia formation was determined by the following equation:

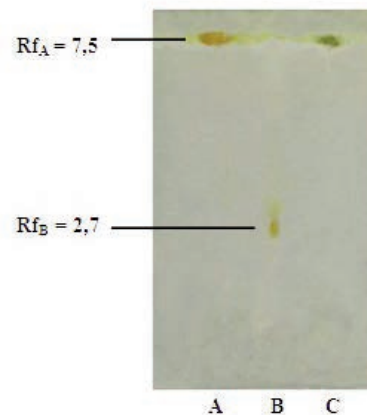


Figure 1. Qualitative alkaloids profile on TLC plates of the 70% ethanol extract (b) and 70%-fractionated ethanol extract (c) of *J. gendarussa* leaves was compared with alkaloids extracts of *Piper nigrum* fruits (a) using Dragendorff spray reagent.

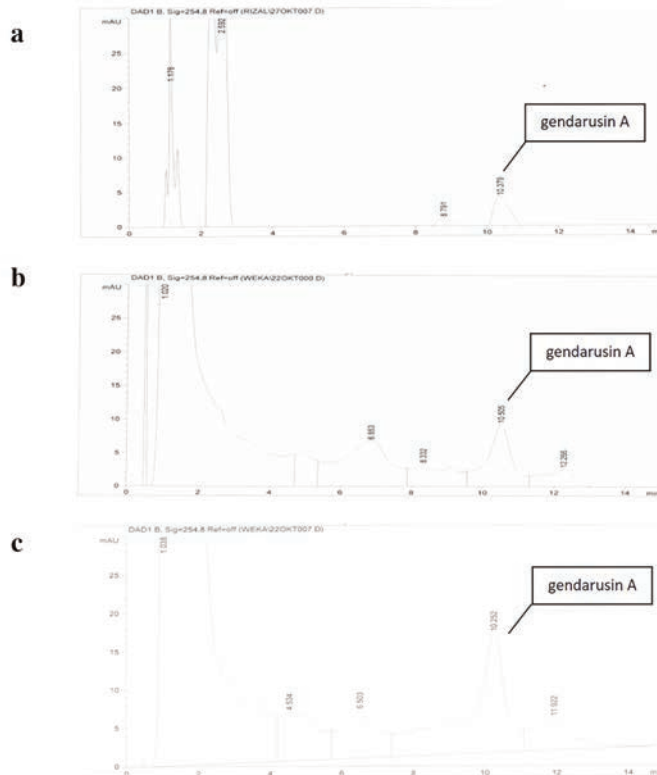


Figure 2. Chromatogram profile of gendarusin A in 12.8 µg/ml solution (a), gendarusin A in 5000.0 µg/ml of the 70%-fractionated ethanol extract of *J. gendarussa* leaves (b), gendarusin A in 5000.0 µg/ml of the 70% ethanol extract of *J. gendarussa* leaves (c).

syncytia formation (%) = $100 - \frac{\text{syncytia number in sample-treated culture}}{\text{syncytia number in untreated culture}} \times 100$

syncytia number in untreated culture

Detection of HIV p24 Antigen

Effect of *J. gendarussa* leaves extracts on HIV replication was assayed by measuring p24 antigen. Level of p24 antigen was used as indicator of HIV infection in HIV-infected MOLT-4 cells. A 200 mL of cell suspension in each well from 96-well micro plate of syncytia assay were transferred into 1.5 mL sterile Eppendorf tube. The cell suspension was inactivated by using 70% ethanol for 1 hour. Then, supernatant of the suspension were collected to determine viral production levels through measurement of HIV p24 antigen level using HIV-1 p24 capture ELISA Kit. The supernatant was plated in each well on a micro titter plate. Then the supernatant was coated with murine anti-HIV-1 p24 monoclonal antibody. HIV-1 p24 antigen from cell culture supernatant was quantified by ELISA (HIV-1 p24 ELISA kit, Xpress-Bio, USA) according to manufacturer's instructions. The lower limit of the assay is 1.7 pg/mL. The amount of captured p24 was determined through measuring the absorbance using micro plate absorbance reader (Bio-Rad) at 450 nm. The p24 content was estimated by Forecast formula in Microsoft Excel 2007 software.

Statistical Analysis

The collected data was analyzed with regression analysis using Microsoft Excel 2007 software. From the regression analysis, the concentration level of *J. gendarussa* leaves extracts that can inhibit of 50% virus growth (50% Effective Concentration, EC₅₀) could be determined. The difference activity of the extracts was considered to be significant the probability was P>0.05.

Results

Alkaloids Analysis

The presence of alkaloids compounds in extracts of *J. gendarussa* was analyzed using TLC method. It was found that the 70% fractionated ethanol extract does not contain alkaloids compounds, while the 70% ethanol extract of *J. gendarussa* leaves contains alkaloids compounds (R_f value = 2.7) (Figure 1).

Gendarusin A Analysis

HPLC chromatograms showed that the 70% fractionated ethanol extract (Figure 2b) and the 70% ethanol extract (Figure 2c)

contain gendarusin A as a major flavonoid component of *J. gendarussa* leaves. The 70% fractionated ethanol extract and 70% ethanol extract of *J. gendarussa* leaves contains 0.53% and 0.95% of gendarusin A, respectively. The results were compared with the alkaloids extracts of *Piper nigrum* fruits (Figure 2a) using Dragendorf spray reagent.

Effect of *J. gendarussa* leaves extracts on syncytia assay

Syncytia formation was significantly inhibited by various concentration of the

70%-fractionated ethanol extract and 70% ethanol extract of *J. gendarussa* leaves (P<0.05). This result could be seen in Figure 3. It was found that *J. gendarussa* leaves extracts were potent drug ability as anti-HIV agent. It was found that EC₅₀ value of the 70%-fractionated ethanol extract of *J. gendarussa* leaves less than 100 µg/ml. Also, it was found that the 70%-fractionated ethanol extract of *J. gendarussa* leaves was more active as anti-HIV agent than 70% ethanol extract of *J. gendarussa* leaves in inhibition of syncytia formation (multinucleate giant cells). This result could be seen in Table 1.

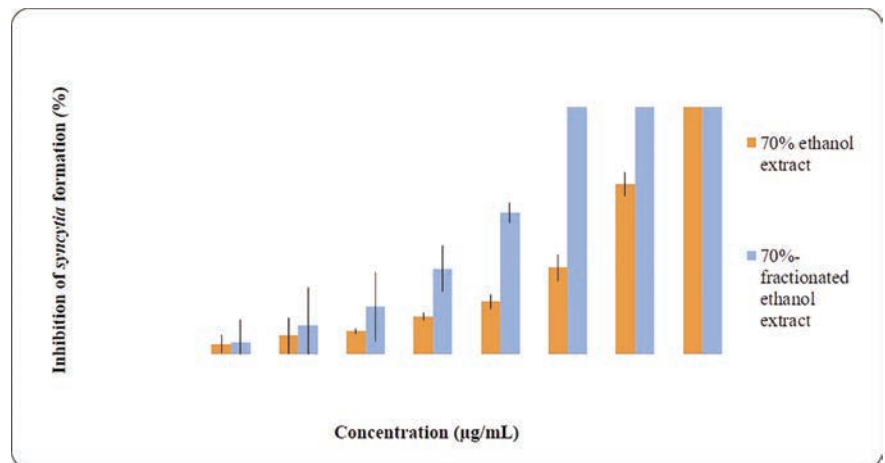


Figure 3. The percentage of syncytia formation inhibition on HIV-infected MOLT-4 cells of 70% fractionated-ethanol extract and 70% ethanol extract from *J. gendarussa* leaves with various concentrations. It was measured after 72 hr incubation under conditions. Error bars show the standard deviation from average of three data.

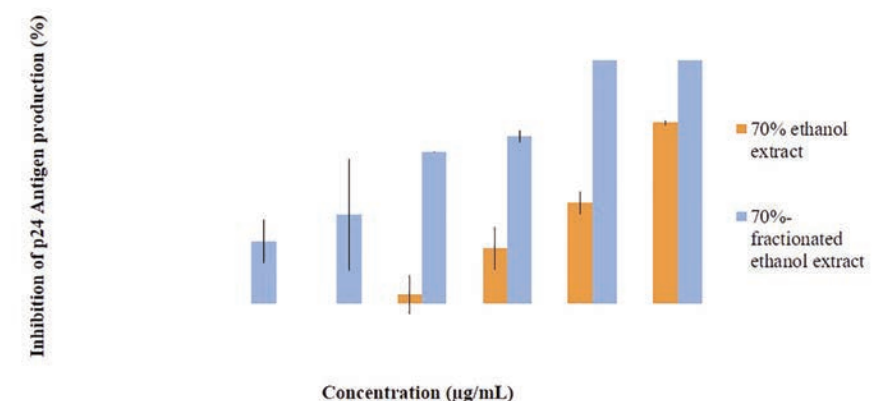


Figure 4. The percentage of p24 antigen expression inhibition on HIV-infected MOLT-4 cells of 70%-fractionated ethanol extract and 70% ethanol extract from *J. gendarussa* leaves with various concentrations. It was measured after 72 hr incubation under conditions. Error bars show the standard deviation from average of three data.

Effect of *J. gendarussa* leaves extracts on HIV p24 antigen expression

By using HIV p24 antigen detection assay, it was found that virus production on MOLT-4 cells exposed by MT-4/HIV was significantly inhibited by various concentration of the 70%-fractionated ethanol extract and 70% ethanol extract of *J. gendarussa* leaves ($P < 0.05$). This result could be seen in Figure 4. The result showed that the 70%-fractionated ethanol extract of *J. gendarussa* leaves was the more active as anti-HIV agent than 70% ethanol extract of *J. gendarussa* leaves by parameter reduction of HIV p24 antigen expression because EC_{50} value of the 70%-fractionated ethanol extract of *J. gendarussa* leaves less than 100 $\mu\text{g/mL}$. It can be seen in Table 1.

Discussion

In this research, MT-4/HIV cell was cultured in the presence and absence of various concentrations of the extracts. Then, the MT-4/HIV cell was exposed to MOLT-4 cell. After 72-hour, syncytia formation was observed and the supernatant were collected. Then, the exposed MOLT-4 cell was subjected to p24 antigen detection assay. Through a series of tests, the corresponding EC_{50} (Effective Concentration 50%) value was determined.

HIV replication in an in vitro cell culture could be observed through the cytopathic effect such as syncytia formation. Also, HIV replication could be monitored through detection of viral products such as p24 antigen.¹⁸ The p24 antigen is an important structural component of retroviral particle. The retroviral particle corresponds with mature virus at the end of HIV replication process. The p24 antigen is a protein capsid surrounding the viral RNA genome. A p24 antigen is still detected in a patient's serum or blood plasma in which its RNA undetectable in an early infection. Detection of p24 antigen could be used in monitoring early treatment of acute infection (early infection). A p24 antigen could be detected outside viral particle or in the supernatant cell culture, while RNA is located inside viral particle.¹⁹

It is well-known that the increasing of viral transcription will normally lead to an increasing of HIV replication. HIV replication is followed releasing of viral particles (such as p24 antigen) from mature virus.¹⁹ During viral replication process, increasing of viral transcription also lead to increasing of production of viral surface proteins (gp120) on the surface of host cells. Interaction of virus surfaces proteins (gp120) with CD4 molecule found on the

surface of uninfected T-cells causes syncytia formation. With the presence of 70%-fractionated ethanol extract of *J. gendarussa* leaves in a viral replication process, viral transcription could be prevented. In addition, production of viral surface proteins on the surface of the host cell is not occurred. Thus, interaction between the virus surfaces proteins with the CD4 molecule found on the surface of uninfected T cells will not occur. As a result, syncytia formation could be inhibited.

The level of anti-HIV activity was indicated by the value of 50% Effective Concentration (EC_{50}). EC_{50} values were determined as concentration of the extracts could inhibit 50% of HIV growth. When EC_{50} value of an extract less than 100 mg/mL , then the extract has an antiviral activity.¹⁸

Based on the result, we found that the two extracts could inhibit the expression of p24 antigen. This fact was evidenced by decreasing of the amount of p24 antigen in MOLT-4 cell cultures were infected with HIV. The reduction of p24 antigen by the extracts leads the inhibition of HIV replication. This means that the extracts could reduce the growth of new viruses in vitro. On the other hand, amount increasing of p24 antigen in a cell culture showed that the higher expression of p24 antigen the in cell culture, the higher syncytia formation in the infected cell cultures is.

Alkaloids need to be removed from the extract because the existence of alkaloids would cause to the vomiting effect. Neurotransmitters, particularly alkaloid compounds, act directly on receptors located in the brain's 4th ventricle, a cavity near the brainstem. When these receptors are activated, nausea results. Some conditions, such as inflammation of the gut, stimulate the brain to induce nausea directly.⁴ Previous research shown that a 70% alkaloids-free ethanol extract of *J. gendarussa* leaves could inhibit reverse transcriptase enzyme. As a result, the transcription process of viral genetic material to form a new virus was inhibited.¹³ The results of this study confirmed the previous results: the presence of a reverse transcriptase enzyme inhibitor will make the transcription process

that changes the viral RNA to be provirus DNA. In addition, presence of the enzyme inhibitor will prevent new virus production.

The difference of EC_{50} value form two extracts might be a consequence of complexity of HIV infection process.²⁰ It might be due to the fact that each extract has slightly different chemical content. So, the extracts efficacy in preventing the virus replication is also different. The preventing of virus replication activity might be due to the presence of useful compounds in the extracts of *J. gendarussa* leaves. Without presence of alkaloids, this extract has anti-HIV activity greater than the extract containing alkaloids. The major flavonoids component in *J. gendarussa* leaves is gendarusin A, which consist of a flavonoid, apigenin glycosides, a flavone group as aglycone, and two arabinose groups, as glycine.¹⁰ Flavonoids of flavonols and flavones groups (such as apigenin) could inhibit the reverse transcriptase enzyme. The general mechanisms in which enzyme activities are affected by inhibitors is well known, including irreversible damage of enzyme active sites or interference with substrate binding.²¹

The diversity of compounds was contained in the 70%-fractionated ethanol extract and 70% ethanol extract of *J. gendarussa* leaves ensures that the extract could work to reduce viral growth by preventing viral replication. The preventing of viral replication might be caused by reverse transcriptase inhibition. In addition, the 70%-fractionated ethanol extract and 70% ethanol extract of *J. gendarussa* leaves also inhibit the viral fusion process. This result was confirmed by syncytia assay. The extracts might contain some compounds that act as good anti-viruses, but the quantity of the compounds is not enough to inactivate all viral particles. Separation of the active compounds in a multilevel extraction method of the plant might be useful to obtain a new and a more effective anti-virus.

Table 1. Anti-HIV activities of ethanol extracts form *J. gendarussa* leaves on HIV-infected MOLT-4 cell clone#8 by syncytia formation and p24 detection assay.

Samples	EC_{50} values	
	Syncytia assay	p24 antigen detection assay
70% ethanol extract of <i>J. gendarussa</i> leaves	228.7 g/mL	540.7 g/mL
70 %-fractionated ethanol extract of <i>J. gendarussa</i> leaves	70.5 g/mL	88.8 g/mL

Note: incubated period for 72 hr.

Conclusions

It was found that 70%-fractionated ethanol extract (with releasing alkaloids) of *J. gendarussahas* has an anti-HIV activity on HIV-infected MOLT-4 cells greater than the anti-HIV activity of 70 % ethanol extract, without releasing alkaloids. This result was shown by syncytia formation assay and HIV p24 antigen assay. It can be concluded that the *J. gendarussa* could be used as a useful resource in developing a phyto-pharmaceutical product that has *in vitro* anti-HIV activity.

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