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ANTIVIRAL ACTIVITY OF *Justicia gendarussa* Burm.f. LEAVES AGAINST HIV-INFECTED MT-4 CELLS

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Abstract

Backgrounds: *Justicia gendarussa* Burm.f. has been known to have anti-HIV activity. This study was conducted to evaluate the effect of incubation time on the antiviral activity of the *J. gendarussa* leaves extract on HIV-infected MT-4 cells *in vitro*. Molecular docking test was also conducted to determine the interaction of alkaloids and flavonoids on the *J. gendarussa* leaves against HIV-1 reverse transcriptase receptor. It is expected that this research will provide scientific information on the development of *J. gendarussa* leaves as an anti-HIV drug.
Materials and Methods: In the activity test, the effect of incubation time on the antiviral activity of *J. gendarussa* leaves on HIV-infected MT-4 cells were evaluated. During the activity test, a parameter of cytolysis effect inhibition on MT-4 cell line was observed after 4 days and 6 days incubation period. The molecular docking test is performed by using Molegro Virtual Docker software to determine the interaction of alkaloid and flavonoid compounds of *J. gendarussa* leaves with HIV-1 reverse transcriptase receptor.
Results: The incubation time influences the CC_{50} and EC_{50} value. Fractionated-70% ethanol extract of *J. gendarussa* leaves showed a higher anti-HIV activity with EC_{50} = 3.045 x 10^{-9} µg/mL, SI = 6.309 x 10^{12} (4 days of incubation) and EC_{50} = 6.066 µg/mL, SI = 58494.845 (6 days of incubation). From molecular docking test, it was found that flavonoid of *J. gendarussa* leaves could inhibit the activity of HIV reverse transcriptase enzyme.
Conclusion: Fractionated-70% ethanol extract of *J. gendarussa* has potential as an anti-HIV.

Keywords: *Justicia gendarussa*, HIV, MT-4 cell, Antiviral

Introduction

Human Immunodeficiency Virus (HIV) is a retrovirus that infects the human immune system. HIV damages the human immune system by destroying T lymphocyte cell (a type of leukocyte). On the T lymphocyte cell surface, there is cluster of differentiation 4(CD4) receptor. When a number of T-CD4 lymphocyte cells are low then a person more susceptible to be infected (Pattman *et al*., 2005). The immune system will break down slowly over the increasing number of viruses (viral load) in the body (Naif, 2013). The final stage of HIV infection is AIDS, a condition when CD4 level decrease to very low level (typically <200 cells/mm^3). This causes ability of the immune system against certain infections seriously impaired (Pattman *et al*., 2005).

One strategy for HIV-AIDS treatment is antiretroviral therapy (ARV). The anti-retroviral therapy is used to disrupt the life cycle of HIV. This therapy is effective for stabilizing viral load in the blood for a long enough time. But the anti-retroviral therapy also has many side effects that could affect various organ systems (Luma *et al*., 2012). The side effect could cause drug resistance and worsen the condition in patients. Therefore, new anti-HIV drugs need to be developed through the discovery of new anti-HIV drugs that are more selective and have low toxicity.

The development of anti-HIV drugs could be performed through the traditional medicine research program both flora and fauna. Traditional medicine research is directed to find scientific evidence on the traditional medicine. Currently, there is one plant that is being developed as anti-HIV, namely *Justicia gendarussa* Burm.f. (Acanthaceae).

J. gendarussa is often used as a traditional medicine. Root and leaves of *J. gendarussa* are usually used as a traditional medicine (Suryowinoto and Pudjoarinto, 1985).

The results of in vitro test showed that 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves have activity in inhibiting HIV reverse transcriptase enzyme (Woradulayapinij *et al*., 2005; Prajogo *et al*., 2016). Current research on *J. gendarussa* leaves extract on MOLT-4 cell cultures infected with HIV showed that *J. gendarussa* leaves could inhibit HIV replication by decreasing the number of p24 and inhibition of *syncytia* formation (Widiyanti *et al*., 2016).

J. gendarussa leaves contain substituted aromatic amine (Chakravarty *et al*., 1982), flavonoid glycoside i.e. gendarusin A and gendarusin B (Prajogo, 2009), and justidrusamida alkaloids A, B, C and D (Kiren *et al*., 2014). The main component of the 70% ethanol extract is apigenin flavonoid glycoside called Gendarusin A (Prajogo, 2014). Flavonoid compounds are known as an anti-HIV natural source of therapy for patients with AIDS by inhibiting HIV reverse transcriptase (Veljkovic *et al*., 2007; Ko *et al*., 2009).

In connection with the evaluation of traditional medicine as an anti-HIV, established guidelines of the World Health Organization (WHO) suggest for doing an in vitro test to detect activity and determine the toxicity potential of the traditional medicine. In vitro test is the first step to identify potential compounds with anti-HIV activity. If the activity is greater than its toxicity, then the traditional medicine can proceed to the next test phase. The next step is confirmation of antiviral activity and toxicity in various cell systems (Organization, 1989).

In this study, the anti-HIV activity of *J. gendarussa* leaves extracts on MT-4 cells was tested. MT-4 cells were selected since the cells are particularly vulnerable to the cytopathic effects of HIV. So the cells are often used in cytotoxicity and antiviral activity test (Kimura *et al*., 1999). Extract of *J. gendarussa* leaves used 70% ethanol extract, and fractionated-70% ethanol extract (free alkaloids), and water extract. Selection of the extract type is based on several previous studies, where use 70% ethanol extract of *J. gendarussa* leaves and water extract of *J. gendarussa* leaves showed potent activity as an anti-HIV (Woradulayapinij *et al*., 2005; Prajogo *et al*., 2016; Widiyanti *et al*., 2016).

Materials and Methods

Plant Materials and Extraction Procedure

Leaves of *J. gendarussa* were obtained from a cultivated crop in Pacet, Mojokerto, East Java province, Indonesia. This plant was identified by the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University under the voucher number 18/H3.1.5/DT/2012. Simplicia powder of *J. gendarussa* leaves was divided into two groups: acidified leaves powder to release alkaloids and non-acidified leaves powder. Both the powder was extracted using 70% ethanol for 3x24 hours in a macerator device, and then the resulting filtrate were concentrated using a rotary evaporator. The extract is dried at 50°C a temperature of to obtain a 70% ethanol extract (17.4% w/w) and fractionated-70% ethanol extract (6.4% w/w) of *J. gendarussa* leaves. Water extract of leaves of *J. gendarussa* is made by blending fresh *J. gendarussa* leaves in cold water. Then the resulted filtrate was collected and was dried using the freeze-dry method to obtain water extracts (1.8% w/w) of *J. gendarussa* leaves.

Alkaloid and Flavonoid Screening of *J. gendarussa* Leaves Extract

Alkaloid screening of 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves be performed using a TLC GF254 plate with mobile phase chloroform: methanol (9:1). Here, Dragendorf reagent is used to reveal stains. Results test is said to be alkaloid positive if there are orange stains.

Flavonoid screening of 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves be performed by using the immobile phase of TLC GF254 with a mobile phase of butanol: glacial acetic acid: water (4:1:5). Here, borate citric is used to reveal stains. Results test is said to be flavonoid acid if there are yellow-green fluorescence stains using UV 366 nm (Indonesia, 2008).

Reagents and Chemicals

Zidovudine-Lamivudine (ZDV/3TC) (Duviral®), 2 µm nitrocellulose membrane filter (Whatman), sterile water for injection, and aquadest were obtained from CRS-EIRD, Institute of Tropical Disease, Surabaya, Indonesia. RPMI-1640 medium and *Fetal Bovine Serum* (FBS) were purchased from Gibco. Natrium bicarbonate and 70% ethanol *pharmaceutical grade* were purchased from Merck. WST-1 was purchased from Roche. Dimethyl sulfoxide (DMSO) was purchased from Sigma.

Cells and Viruses

The MT-4 cells (human T-cell leukemia line) were obtained from the Institute of Tropical Disease (ITD) laboratory, Airlangga University, Surabaya, Indonesia. MT-4 cells were cultured in RPMI-1640 media and were equipped with 10% FBS. The MT-4 cells were maintained in T_{25} CCF at 37 °C temperature in a 5% CO_2 incubator. HIV isolates from a seropositive HIV donor that labeled IDU-18 obtained from ITD laboratory, Airlangga University, Surabaya, Indonesia. HIV cultured on the MT-4 cell in RPMI-1640 medium completed with FBS 10%. MT-4/HIV cell kept in CCF T_{25} at 37 °C in CO_2 5% incubator.

Cytotoxicity Test

The cytotoxicity test was performed by a colorimetric method using WST-1 (Kangro and Mahy, 1996; Zhang *et al*., 2012). 50 µl MT-4 cells in a 96-*well microplate* (2 x 10^5 cells/well) in the absence or presence of various concentrations of *J. gendarussa* leaves extracts were incubated for 4 days and 6 days at 37°C temperatures in a 5% CO_2 incubator. Then 10 µl WST-1 was added to each well and was incubated for 2 hours at 37 ° C temperature in a 5% CO_2 incubator. ZDV/3TC was used as a positive control. The absorbance was measured at 450 nm wavelength using a microplate absorbance reader.

Cytolysis Effect Inhibitory Test

The cytolysis effect inhibitory test was performed by a colorimetric method using WST-1 (Kangro and Mahy, 1996; Zhang *et al*., 2012). MT-4 cells (2 x 10^5 cells/well) were infected with HIV in the absence or presence of various concentrations of *J. gendarussa* leaves extracts were incubated for 4 days and 6 days at 37°C temperatures in a 5% CO_2 incubator. Then 10 µl WST-1 was added to each well and was incubated for 2 hours at a 37°C temperature in a 5% CO_2 incubator. ZDV/3TC was used as a positive control. The absorbance was measured at 450 nm wavelength using a microplate absorbance reader.

Statistical Analysis

Data is analyzed by probit regression analysis using the Minitab version 17 software. Probit regression analysis is used to determine 50% *Cytotoxicity Concentration* (CC_{50}), which is a concentration that able to decrease 50% of cell viability.

Molecular Docking Test

The molecular docking test is performed to determine the interaction of an alkaloid and flavonoid compounds of *J. gendarussa* leaves with HIV-1 reverse transcriptase receptor. The structure of the alkaloid and flavonoid compound is determined by ChemBioOffice Ultra version 12.0 software. The structure of the HIV-1 reverse transcriptase receptor is obtained from Protein Data Bank (http://www.pdb.org/pdb/home/home.do) with 3V4I code. Molecular docking analysis is performed by using Molegro Virtual Docker (MVD) version 5.0 software. From molecular docking test, it obtains the rerank score. Then the rerank score is used as activity prediction.

Results

TLC performed the alkaloid screening (Table 1). It was found that some oranges stain in 70% ethanol extract and water extract of *J. gendarussa* leaves. It indicates that in both the extracts contain alkaloids. From the flavonoid screening result, it was found that greenish yellow fluorescence stain in 70% ethanol extract, fractionated-70% ethanol extract, and water extract of *J. gendarussa* leaves. It indicates that the three extracts contain flavonoids.

Table 1: Alkaloid and flavonoid screening of the *J. gendarussa* leaves extract

Extract	Test	
	Alkaloid	**Flavonoid**
70% ethanol extract	+	+
fractionated-70% ethanol extract	-	+
water extract	+	+

(+) Detected; (-) Not Detected

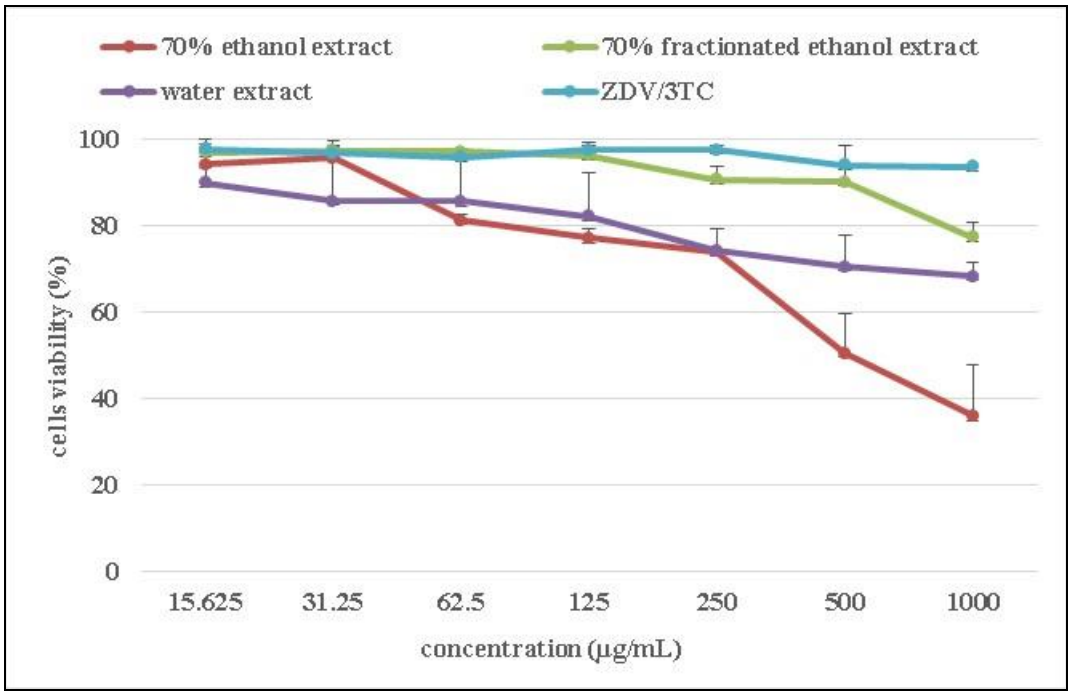

Figure 1: Effect of extracts of *J. gendarussa* leaves and ZDV/3TC on the viability of MT-4 cells after 4 days incubation period.

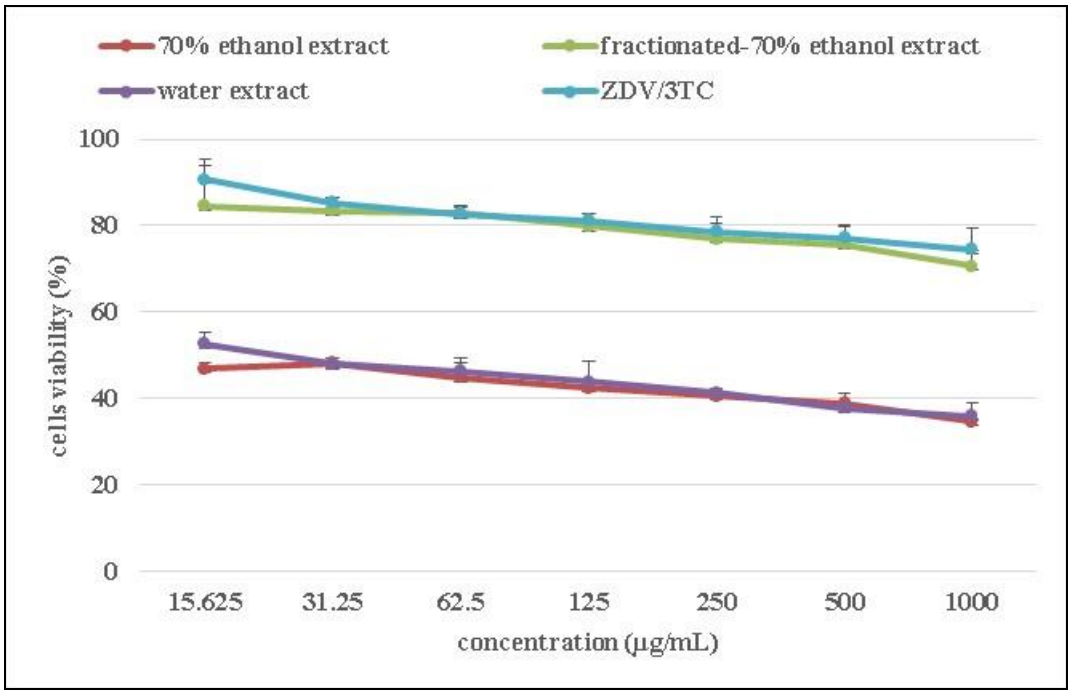

Figure 2: Effect of extracts of *J. gendarussa* leaves and ZDV/3TC on the viability of MT-4 cells after 6 days incubation period.

From the cytotoxicity test, it was found that incubation period affects cytotoxicity of *J. gendarussa* leaves extract in MT-4 cells. Increasing concentrations extracts of *J. gendarussa* leaves could reduce MT-4 cells viability. The viability of MT-4 cells after 4 days incubation period and 6 days incubation period were shown in Figure 1 and Figure 2 respectively. The value of cell viability was used to determine 50% cytotoxicity concentration (CC_{50}) value for each test material. Probit regression analysis has calculated the value of CC_{50}. The probit regression analysis was performed by using Minitab software. The CC_{50} value was presented in Table 2.

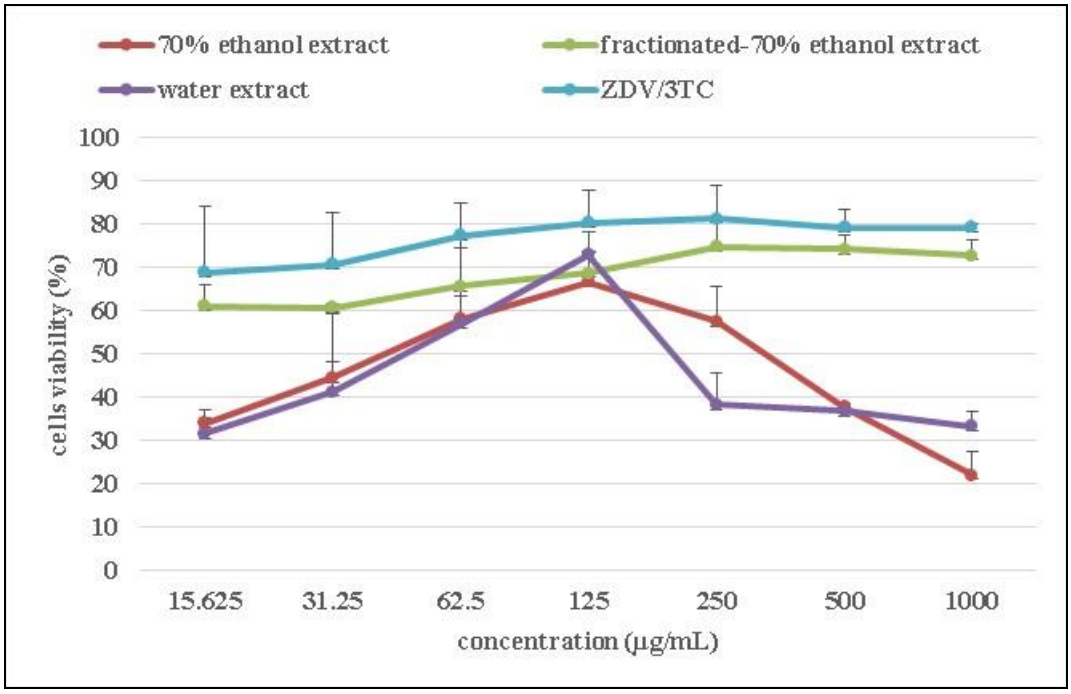

Figure 3: The Cytolysis inhibition effect of extracts of *J. gendarussa* leaves and ZDV/3TC on MT-4 cells viability were infected with HIV after 4 days incubation period.

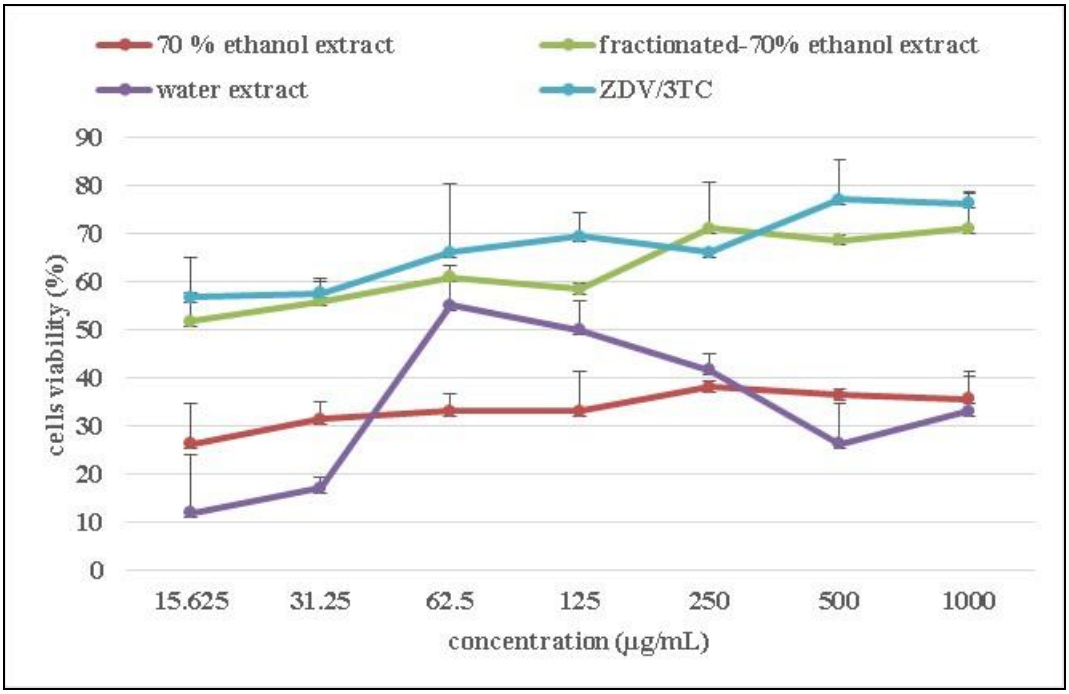

Figure 4: The Cytolysis inhibition effect of extracts of *J. gendarussa* leaves and ZDV/3TC on MT-4 cells viability were infected with HIV after 6 days incubation period.

From cytolysis inhibition test, it was found that incubation time affects the anti-HIV activity of *J. gendarussa* leaves extract on MT-4 cells (Figure 3 and Figure 4). Increasing of ethanol extract concentration of *J. gendarussa* could affect the viability of MT-4 cells infected with HIV. Then, the percentage of the MT-4 cells viability was used to determine the 50% effective concentration (EC_{50}) value of each test material. Probit regression analysis has calculated the EC_{50} value. The probit regression analysis was performed by using Minitab software. The EC_{50} value was presented in Table 2.

Table 2: 50% effective concentration (EC_{50}), 50% cytotoxicity concentration (CC_{50}), and Selectivity Index on MT-4 cell cultures after 4 days and 6 days incubation period

	CC_{50} (μg/mL)		EC_{50} (μg/mL)		SI	
Time of Incubation	**4 days**	**6 days**	**4 days**	**6 days**	**4 days**	**6 days**
EA	1123.414	19.295	29853.826	7289.706	0.037	0.003
FEA	19211.001	354829.729	3.045×10^{-9}	6.066	6.309×10^{12}	58494.845
WE	2293.454	26.541	187616.055	6861.831	0.012	0.004
ZDV/3TC	67956.343	807290.794	1.160×10^{-14}	1961	5.858×10^{18}	411673.021

EA: 70% ethanol extract of *J. gendarussa* leaves; FEA: fractionated-70% ethanol extract of *J. gendarussa* leaves; WE: Water extract of *J. gendarussa* leaves; ZDV/3TC: Zidovudine-Lamivudine; SI: Selectivity Index= CC_{50}/EC_{50}.

Molecular docking test results (Table 3), it was presented the same chemical bonds among the amino acids of HIV-1 reverse transcriptase (3V4I) with flavonoids and alkaloids of *J. gendarussa* leaves and Zidovudine.

Table 3: Rerank score and hydrogen bonding interactions between receptors of HIV-1 reverse transcriptase (3V4I) with chemical compounds of *J. gendarussa* leaves

Class	**Chemical Compound**	**Amino Acid**			**Rerank Score**
		Tyr 115	**Asp 113**	**Lys 65**	
NRTIs	Zidovudine	+	+	+	-85.9473
Alkaloid	2_(2_Amino_benzylamino)_methyl_benzyl alcohol	-	+	-	-71.1579
	2_(2_Amino_benzylamino)_O_methyl_benzyl alcohol	-	-	-	-71.9826
	2_Amino_O_methyl_benzyl alcohol	-	+	-	-52.3556
	2_Aminobenzyl alcohol	-	-	-	-48.7308
	Justidrusamide AB	-	-	-	-82.4664
	Justidrusamide CD	+	-	-	-84.0718
Flavonoid	Gendarusin A	+	-	-	-94.7381
	Gendarusin B	+	+	-	-83.6573
	Gendarusin C	+	-	+	-106.808
	Gendarusin D	-	-	-	-91.3127
	Gendarusin E	+	-	-	-101.094

NRTIs: nucleoside reverse transcriptase inhibitors

Discussion

Cytotoxicity testing with incubation time variation was performed to determine the effect of incubation time against the cytotoxicity potential of *J. gendarussa* leaves extract on MT-4 cells. Cytotoxicity testing was performed by a colorimetric method using a WST-1 test. The test was based on the reduction of the WST-1 by live cells produce soluble formazan (Rode, 2008). The amount of formed formazan represents high cell viability, which is the numbers of active living cells metabolize WST-1 (Rampersad, 2012).

Observation of cytotoxicity activity was performed by calculating the percentage of MT-4 cells viability. Then a regression equation between the percentage of cell viability and the logarithm of concentration was determined. The CC_{50} value determined the amount of cytotoxicity activity of the extract. The CC_{50} value of an extract is the extract concentration of that able to decrease 50% of cell viability. In this study, cytotoxicity activity was observed on different days, namely on the fourth day and sixth day, because previous studies reported that incubation time differences caused a significant difference in CC_{50} values (Kimura *et al*., 1999). From this study, there were differences of CC_{50} value between the fourth and the sixth day, especially for the 70% ethanol extract and water extract of *J. gendarussa* leaves where the CC_{50} was not toxic at the fourth day and then become more toxic in the sixth day. It is apparently due to the compound content of the extract. The presence of alkaloid compounds seems to give a major influence on cell death when it's compared to the flavonoid compounds (Astuti *et al*., 2006). The influence of alkaloids against cell death could be identified from the results of 70% ethanol extract and water extract of *J. gendarussa* leaves (Figure 2), where on the sixth day of incubation almost of all concentrations causes the MT-4 cell viability less than 50%. Meanwhile, the results of fractionated-70% ethanol extract (free alkaloids) show the cell viability greater than 70% after 6 days incubation period.

In the anti-HIV activity test, we used inhibitors of cytolysis effect of 70% ethanol, fractionated-70% ethanol extract and water extract of leaves *J. gendarussa* against MT-4 cell cultures infected with HIV after 4 days and 6 days incubation period as a parameter of anti-HIV activity. The results of the previous study stated that incubation of MT-4 cells with MOLT-4 / HTLV-IIIB (strains of HIV-1) for 4 days led to more than 95% cells contain viral antigen (Yang *et al*., 1996). Also, the incubation time difference caused a significant difference in EC_{50} and CC_{50} values (Kimura *et al*., 1999). Hence, 4 days and 6 days incubation period were chosen in this research.

This study found that 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves have an activity of cytolysis effect inhibiting on the MT4 cell with infected by HIV both at fourth day and sixth day incubation period. The EC_{50} value of a test sample represents the concentration of the test sample which can inhibit 50% of viral replication in a cell-based testing. An extract is said to have anti-HIV activity when the

extract has EC_{50} below 100 µg/mL (Cos *et al*., 2006). From the EC_{50} values, the fractionate-70% ethanol extract of *J. gendarussa* leaves has anti-HIV activity by inhibiting the cytolysis effect on HIV-infected MT-4 cells with an EC_{50} value below 100 µg/mL. Meanwhile, 70% ethanol extract and water extract of *J. gendarussa* leaves has lower anti-HIV activity since their EC_{50} value is greater than 100 µg/mL.

The CC_{50} and EC_{50} values could be used to determine the Selectivity Index (SI) of anti-HIV activity of *J. gendarussa* leaves extract. The SI is used as a parameter which indicates toxicity of *J. gendarussa* leaves extract to cell and virus. The CC50 value should be greater than EC_{50} value. It was found that SI of fractionated-70% ethanol extract much higher than the 70% ethanol extract and water extract of *J. gendarussa* leaves. The greater SI, the greater potential of a test material as a drug (Kimura *et al*., 1999; Volberding, 2008).

Molecular docking test results (Table 3) indicate that flavonoid compounds in *J. gendarussa* leaves and Zidovudine bind same amino acids. The rerank score of the flavonoid and Zidovudine are low. The rerank score of a compound represents its bond energy. Hence, the rerank score is used to predict the flavonoid and Zidovudine activity. Bond energy is the energy required for ligand binding to its receptor. The smaller the binding energy, more stable and easier a ligand binds its receptor will be. The more stable a ligand is binding a receptor, it could be predicted that the greater its activity will be higher (Thomsen and Christensen, 2006). The results of this research showed that fractionated-70% ethanol extract of *J. gendarussa* has potential as an anti-HIV. In future, *J. gendarussa* could be used as a potential candidate for an anti-HIV drug.

Conflict of Interest: The authors declare that there are no conflicts of interest.

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