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EFFECT OF VARYING INCUBATION PERIODS ON CYTOTOXICITY AND VIRUCIDAL ACTIVITIES OF *Justicia gendarussa* Burm.f. LEAF EXTRACT ON HIV-INFECTED MOLT-4 CELLS

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Abstract

Backgrounds: *Justicia gendarussa* Burm.f. has an anti-HIV activity. This study was conducted to evaluate the effects of incubation periods on the cytotoxicity and virucidal activities of the *J. gendarussa* leaves extract on MOLT-4 cells.

Materials and Methods: The cytotoxicity assay was evaluated by using the WST-1 test with incubation periods of 3 days and 5 days. The virucidal activity test was determined by measuring the inhibitory activities on the syncytium formation.

Results: The cytotoxicity assay showed the value of CC₅₀ on MOLT-4 cell culture with the test material of 70% ethanol extract of *J. gendarussa* leaves as much as 3928.620 µg/mL and 3176.581 µg/mL (incubation day 3 and day 5, respectively); fractionated-70% ethanol extract = 81782.428 µg/mL and 12175.870 µg/mL; and water extract = 16372.689 µg/mL and 2946.117 µg/mL. The test results of the virucidal activities (inhibit ≥ 90% the formation of syncytium) of 70% ethanol extract of *J. gendarussa* leaves is at a concentration 250 µg/mL, 500 µg/mL and 1000 µg/mL (3-day incubation) and 250 µg/mL (5-day incubation); and fractionated-70% ethanol extract at a concentration 250 µg/mL, 500 µg/mL and 1000 µg/mL (3-day incubation) and 1000 µg/mL (5-day incubation).

Conclusion: 70% ethanol extract, fractionated-70% ethanol extract, and water extract of *J. gendarussa* leaves were relatively nontoxic toward MOLT-4 cells, and fractionated-70% ethanol extract had better potentials in virucidal activities.

Keywords: *Justicia gendarussa* Burm.f., MOLT-4 cells, cytotoxicity, virucidal, incubation periods

Introduction

Justicia gendarussa Burm.f. (Acanthaceae) is often used as traditional medicine. Parts of the plant that are commonly used are the root and leaves. *J. gendarussa* leaves have several benefits, one of which is an antiviral. The results of in vitro tests, 70% ethanol extract, fractionated-70% ethanol extract, and water extract of *J. gendarussa* leaves showed activities inhibiting HIV's reverse transcriptase enzyme (Woradulayapinij et al., 2005; Prajogo et al., 2016).

J. gendarussa leaves contain substituted aromatic amine (Chakravarty et al., 1982), flavonoid glycosides such as gendarusin A and B (Prajogo et al., 2014), and alkaloids of justidrusamides A, B, C and D (Kiren et al., 2014). The main component in the 70% ethanol extract is apigenin flavonoid glycoside called Gendarusin A (Prajogo et al., 2009). Flavonoid compounds act as a natural source of anti-HIV therapy for patients with AIDS by inhibiting HIV's reverse transcriptase (Veljkovic et al., 2007; Ko et al., 2009). Flavonoid compounds in high concentrations can be cytotoxic which causes an increase in the mitochondrial permeability, release of cytochrome c, activation of caspase, increased levels of p53 and p21, bcl-2 pressing, apoptosis induction, and the death of necrosis cell (Bolton et al., 2000; Inayat-Hussain et al., 2001; Morin et al., 2001; Salvi et al., 2002; Shen et al., 2004; Lee et al., 2011). Alkaloid compounds are toxic to humans, but most of them have physiological activities that are prominent and can be widely used in the treatment (Harborne, 1987).

A cytotoxicity study of the *J. gendarussa* leaves extract was conducted to ensure the safety. Cytotoxicity of 70% ethanol extract and fractionated-70% ethanol extract on MOLT-4 cells with 3-day incubation using colorimetric methods with reagent WST-1 showed relatively non-toxic results with CC₅₀ > 41 µg/mL (Widiyanti et al., 2016).

Studies on antiviral activities of *J. gendarussa* leaves extract and water extract of *J. gendarussa* leaves have shown their potential as an anti-HIV. 70% ethanol extract of *J. gendarussa* leaves demonstrated anti-HIV activities by decreasing the amount of p24 antigen and holding back both syncytium formation and reverse transcriptase enzyme (Prajogo *et al.*, 2016; Widiyanti *et al.*, 2016). Water extract of *J. gendarussa* leaves also showed the inhibition ratio of HIV-1 reverse transcriptase of more than 90%, where the inhibition rate was higher than the ethanol extract (Woradulayapinij *et al.*, 2005).

Virucidal activity test needs to be done to verify the ability of the *J. gendarussa* leaves extract in terminating the HIV-1 virus. This test is to determine the ability of the *J. gendarussa* leaves extract in disabling HIV particles. Test results on *Justicia reptans* extracts showed virucidal activities on HIV, which were related to its major components, flavonoid glycosides (Bedoya *et al.*, 2008). Some plant substances such as tannins and phenolic are known to have virucidal effects. These substances interact with a protein of viral particles to reduce and prevent adsorption of the virus to the host cell (Singh *et al.*, 2005).

In this research cytotoxicity assay and virucidal activity assay were conducted with varying incubation periods to determine the effect of incubation period on the cytotoxicity's potential and virucidal activities of the *J. gendarussa* leaves extract on MOLT-4 cells. As a result, information regarding the safety and efficacy of *J. gendarussa* leaves extract in its development as an anti-HIV was obtained.

7 Materials and Methods

Plant

2 *J. gendarussa* leaves were obtained from plants cultivated in the area of Pacet, Mojokerto, East Java, Indonesia. This plant was identified by the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University (voucher no. 18/H3.1.5/DT/2012).

Extraction Procedure

J. gendarussa leaves powder was divided into two groups. The first group was leaves powder acidified to release the alkaloids, and the second one group was the leaves powder that was not acidified. Then both were extracted using 70% ethanol for 3x24 hours in a macerator device, and then the filtrate from the extraction was concentrated using a rotary evaporator. The extract was dried at a temperature of 50°C 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 (1.8% w/w) was obtained by blending fresh *J. gendarussa* leaves into cold water, and then the filtrate was collected and dried by using the freeze dry method.

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

Alkaloids on 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves were screened by using a GF254 TLC plate with chloroform mobile phase: methanol (9:1), with staining using Dragendorff reagent. The alkaloid test results will be positive if there are orange stains.

Flavonoids on 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves were screened by using GF254 TLC stationary phase with a mobile phase of butanol-glacial acetic acid-water (4: 1: 5), with staining using citric borate. Flavonoid test results will be positive if there are yellow-green fluorescent stains using UV 366 nm (Indonesia, 2008).

Materials

70% Ethanol pharmaceutical grade, Methanol pro HPLC (Merck), sterile water for injection, distilled water (pure water) obtained from Lab CRC-EIRD, ITD Surabaya, RPMI-1640 medium (Gibco), sodium bicarbonate (Merck), Fetal Bovine Serum (FBS) (Gibco) inactivated at a temperature of 56°C for 30 minutes, reagent 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene Disulfonate (WST-1) (Roche), dimethyl sulfoxide (DMSO) (Sigma), 0.2 µm nitrocellulose membrane filter (Whatman), and Zidovudine+Lamivudine / ZDV+3TC (Duviral®).

Cells and Viruses

Cell line MOLT-4 clone 8 (Human T lymphocyte cancer cell line) and HIV isolates from a seropositive HIV donor that labeled IDU-18 were obtained from the Institute of Tropical Disease (ITD) laboratory, Airlangga University, Surabaya, Indonesia. MOLT-4 cells were cultured in RPMI-1640 media and equipped with 10% FBS. Cells were maintained in CCF T₂₅ at a temperature of 37°C in 5% CO₂ incubator.

Cytotoxicity Assay

Cytotoxicity from the extract on MOLT-4 cells was tested by a colorimetric method using a WST-1 reagent (Kangro and Mahy, 1996). In short, 50 µL of MOLT-4 cells at a concentration of 2 x 10⁵ cells/well on a plate in each

well in a 96-well microplate. Then, 50 μL of each test solution with varying concentrations (31.25; 62.5; 125; 250; 500; and 1000 $\mu\text{g}/\text{mL}$) was added to each well and incubated for 3 days and 5 days at 37°C in 5% CO₂ incubator. ZDV+3TC was used as a positive control. Control cells without treatment and control of the media were also tested. The total volume per well was 100 μL . After incubation, 10 μL WST-1 reagent was added into each of the wells and incubated for 2 hours at 37°C in 5% CO₂ incubator. Absorbance was measured at a wavelength of 450 nm by using a microplate absorbance reader (Bio-Rad). The high absorbance read indicated the great amount of formazan formed, illustrating that the living cells are highly capable of metabolizing WST-1 salt. The percentage of cell viability was determined by the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{treatment absorbance} - \text{media control absorbance}}{\text{cell control absorbance} - \text{media control absorbance}}$$

Virucidal Test

Virucidal activity testing on the *J. Gendarussa* leaves extract was determined by measuring the inhibitory activity against the formation of the syncytium (Kangro and Mahy, 1996; Tello *et al.*, 2012). In short, 96-well microplates were filled with 100 μL growth culture medium into each well (except for row A). 200 μL of test solution with a concentration of 1000 $\mu\text{g}/\text{mL}$ was added into row A (except the negative control column). Each test treatment group and negative control (without test solution) was done by applying two replications. Dilution was conducted to obtain various concentrations. Then, 50 μL of HIV with a concentration 2×10^4 cells/mL was added to each well containing the test solution. Then, the microplates were put in a 5% CO₂ incubator at a temperature of 37°C and incubated for 30 minutes. After the incubation, 96-well microplates were removed from the incubator, and 50 μL of cells MOLT-4 with a concentration 4×10^5 cells/mL was added to each well. Cytopathic effect was observed on days 3 and 5. The cytopathic effect, the formation of the syncytium, the virus with and without the *J. gendarussa* leaves extract were compared. The inhibition percentage of syncytium formation was calculated by using the following formula:

$$\text{Syncytia Inhibition (\%)} = 100 - \frac{\text{total number of syncytia in the cells with test samples}}{\text{total number of syncytia in the negative control}} \times 100\%$$

Statistical Analysis

Data analyzed by using probit regression analysis used Minitab 17 program to calculate the value of 50% cytotoxicity concentration (CC₅₀), namely the concentration that could lower 50% of cell viability.

Results

Results of screening by TLC (Table 1) alkaloid showed orange stains in 70% ethanol extract and water extract of *J. gendarussa* leaves, indicating that they contained alkaloids. At the flavonoid screening results, greenish yellow fluorescence stains were found in 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves, indicating that they contained flavonoids.

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

Extract	Alkaloid	Flavonoid
70% ethanol extract	+	+
Fractionated-70% ethanol extract	-	+
Water extract	+	+

(+) Detected; (-) Not Detected

Cytotoxicity test results showed that the *J. gendarussa* leaves extract had low cytotoxicity in the MOLT-4 cells both after 3-day and 5-day incubations. The increase of concentrations of each ethanol extract of *J. gendarussa* leaves could lower the percentage of MOLT-4 cell viability (Table 2).

Table 2: Results of cytotoxicity assay in MOLT-4 cell culture after 3 and 5day incubations

Concentration (µg/mL)	Cell Viability (%)							
	EE		FEE		WE		ZDV+3TC	
	3 days	5 days	3 days	5 days	3 days	5 days	3 days	5 days
1000	59.680	51.597	79.135	64.742	67.293	67.293	91.259	91.259
500	59.962	55.528	81.579	67.813	70.771	70.771	91.447	91.447
250	73.120	63.514	84.586	68.305	78.477	78.477	91.823	91.823
125	74.342	66.339	86.936	69.656	79.793	79.793	94.549	94.549
62.5	76.410	70.147	89.944	74.447	84.586	84.586	95.019	95.019
31.25	78.853	76.781	90.320	76.781	88.346	88.346	97.086	97.086

EE: 70% ethanol extract of *J. gendarussa* leaves; FEE: fractionated-70% ethanol extract of *J. gendarussa* leaves; WE: Water extract of *J. gendarussa* leaves; ZDV/3TC: Zidovudine-Lamivudine

The percentage of cell viability was then used to determine the values of CC₅₀ of each test material. CC₅₀ values were calculated by probit regression analysis using Minitab 17 software. The CC₅₀ value was presented in Table 3.

Table 3: CC₅₀ in MOLT-4 cell culture after 3 and 5day incubations

Extract	CC ₅₀ (µg/mL)	
	3day incubation	5day incubation
70% ethanol extract	3928.620	3176.581
Fractionated-70% ethanol extract	81782.428	12175.870
Water extract	16372.689	2946.117
ZDV+3TC	1154489.530	462711.187

The test results of virucidal activities (Table 4) were calculated by the inhibition percentage of syncytium formation performed at various concentrations. Table 4 shows that the increase of the concentration of *J. gendarussa* leaves extract was able to increase the inhibition percentage of syncytium formation in MOLT-4 cells infected with HIV.

Table 4: Test results of inhibition of syncytium formation in MOLT-4 cell culture after 3 and 5 day incubations

Concentration (µg/mL)	Syncytia Inhibition (%)							
	EE		FEE		WE		ZDV+3TC	
	3 days	5 days	3 days	5 days	3 days	5 days	3 days	5 days
1000	100	cd	96.534	91.497	68.803	cd	97.479	94.485
500	100	cd	96.218	88.510	48.004	56.798	96.218	91.497
250	95.903	91.727	94.958	85.063	15.231	22.788	95.903	81.386
125	13.341	31.750	49.895	52.202	9.559	22.328	92.437	78.169
62.5	7.983	30.142	44.223	47.376	10.820	11.528	91.807	80.927
31.25	10.820	2.566	39.181	38.874	4.517	3.485	94.643	60.245

EE: 70% ethanol extract of *J. gendarussa* leaves; FEE: fractionated-70% ethanol extract of *J. gendarussa* leaves; WE: Water extract of *J. gendarussa* leaves; ZDV/3TC: Zidovudine-Lamivudine; cd: cell death

Discussion

Cytotoxicity assay with the variations of incubation periods was performed to determine the effect of incubation periods on the cytotoxicity potentials of *J. gendarussa* leaves extract on MOLT-4 cells. Cytotoxicity assay is the colorimetric method using a tetrazolium salt reagent 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene Disulfonate or better known as the WST-1 assay. The test is based on the reduction of the WST-1 by living cells that produce soluble formazan salt (Rode, 2008). The great amount of formazan formed reflects the high cell viability, namely the number of active living cells metabolizing WST-1 (Rampersad, 2012).

The observation of cytotoxic activities was done by calculating the percentage of MOLT-4 cell viability, and then a regression equation between the percentage of cell viability with log concentration was made. The number of cytotoxicity activities of the extract was determined by the CC₅₀ values in which the concentration of the extract was able to lower 50% of cell viability.

The extract cytotoxicity level was evaluated based on the CC₅₀ values. CC₅₀ values below 20 µg/mL would mean cytotoxic; CC₅₀ values ranging between 21-40 µg/mL would mean low cytotoxic; CC₅₀ values of more than 41 µg/mL would mean non-cytotoxic (Mohamed *et al.*, 2000; Rohaya *et al.*, 2005; Ayob *et al.*, 2013). In this study, observation cytotoxic activity was observed on different days, namely on day 3 and 5, because previous studies have reported that there was a significant difference from the values of CC₅₀ when calculated on different days (Kimura *et al.*, 1999).

A calculation used the probit regression analysis showed that the values of CC₅₀ in MOLT-4 cell culture by incubation period of 3 and 5 days with the test materials 70% ethanol extract, fractionated-70% ethanol extract, and water extract of *J. gendarussa* leaves demonstrated that all *J. gendarussa* leaves extract showed non-cytotoxic activities in MOLT-4 cells because of the values of CC₅₀ > 41 µg/mL (Table 3). Previous research on the cytotoxic activities of 70% ethanol extract and fractionated-70% ethanol extract of *J. gendarussa* leaves on MOLT-4 cells by an incubation period of 3 days using the WST-1 colorimetric method stated that CC₅₀ values obtained from the two extracts also indicated non-toxicity (Widiyanti *et al.*, 2016).

The differences CC₅₀ values between incubation 3 and 5 days were probably due to the compounds of both extracts. The content of alkaloids probably gave a major influence on the cell death when compared to the content of flavonoids in the extracts (Astuti *et al.*, 2006). CC₅₀ values of 70% ethanol extract and water extract were lower than fractionated-70% ethanol extract. This is because the 70% ethanol extract and water extract did not release the alkaloids, which means that the extracts contained a high level of alkaloids (Table 1). Therefore, it gave an effect on MOLT-4 cell death.

The viability of cells in MOLT-4 cell culture showed a decline along with the increase in the concentration of 70% ethanol extract, fractionated-70% ethanol extract, and water extract of *J. gendarussa* leaves. A decrease in cell viability means that there was an inhibition of cell growth associated with suppression of cell proliferation activities so that the number of dividing cells or the living ones declined. Various activities of cell signaling that involved protein expression on programmed cell death such as bcl-2 and bax were likely to be activated when the cell line was exposed to the active compound contained in the extracts (Singh, 2014). An assay using tetrazolium could only detect the formation of formazan (related to the activity of mitochondria of living cells) but not determine the cause of cell death (Paul and Manjula, 2014).

In this study, virucidal activities were also tested with the inhibition parameters of syncytium formation (Table 4). The test materials are considered to have virucidal activities if they inhibit the formation of syncytium ≥90% (Tello *et al.*, 2012). Based on the calculation of the inhibition percentage of syncytium formation in MOLT-4 cultured cells on day 3, test materials that inhibited the formation of syncytium were ≥90% in 70% ethanol extract of *J. gendarussa* leaves at a concentration of 250 µg/mL, 500 µg/mL, and 1000 µg/mL; fractionated-70% ethanol extract of *J. gendarussa* leaves at a concentration of 250 µg/mL, 500 µg/mL and 1000 µg/mL; and ZDV+3TC at a concentration of 31.25 µg / mL, 62.5 µg /mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL. Meanwhile, from the inhibition percentage of syncytium in MOLT-4 cell culture on day 5, it was found that test materials inhibiting the formation of syncytium were ≥90% in 70% ethanol extract of *J. gendarussa* leaves at a concentration of 250 µg/mL; fractionated-70% ethanol extract of *J. gendarussa* leaves at a concentration of 1000 µg /mL; and ZDV+3TC at a concentration of 500 µg /mL and 1000 µg /mL.

Virucidal activity test results showed that the fractionated-70% ethanol extract had better potentials in virucidal activities than other extracts. In fact, 70% ethanol extract of *J. gendarussa* leaves also have the potentials, but it caused death in MOLT-4 cells at the concentration of 500 µg/mL and 1000 µg /mL on day 5 of incubation. Cell death also occurred in the water extract of *J. gendarussa* leaves at a concentration of 1000 µg/mL. This is likely due to the alkaloid content in 70% ethanol extract and water extract of *J. gendarussa* leaves (Table 1).

Differences in virucidal activities from each test material were because each of the test materials has different chemical contents that could inhibit the development phase of the virus differently, such as the stage of viral entry until the stage of replication (Leteane *et al.*, 2012). In *J. gendarussa* leaves extract, without alkaloids, the extracts were capable of providing anti-HIV activities better and lowered the cellular toxicity, compared to the extracts containing alkaloids.

Conclusion

The results obtained showed that 70% ethanol extract, fractionated-70% ethanol extract and water extract of *J. gendarussa* leaves were relatively nontoxic toward MOLT-4 cells after 3 and 5 days incubation. The results of the virucidal activity test showed that fractionated-70% ethanol extract had better potentials in virucidal activities than other extracts.

11

Conflicts of Interest: The authors declare no conflict of interest.

25

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