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Research Report

CYTOTOXICITY OF *JUSTICIA GENDARUSSA* BURM F. LEAF EXTRACTS ON MOLT-4 CELL

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ABSTRACT

Justicia gendarussa Burm f. (Acanthaceae) is known for its activity as a male contraceptive and anti-HIV properties. The present study was designed to evaluate extracts of J. gendarussa for cytotoxicity activity against MOLT-4 cells. The cytotoxic activity of the fractionated-extract and 70% ethanol extracts of J. gendarussa leaves on MOLT-4 cells were evaluated using a WST-1 assay. The treatment cells, control cells without treatment and control media were also tested in duplicate. The absorbance was measured at a wavelength of 450 nm using a microplate absorbance reader (Bio-Rad). The average absorbance measures formazan produced by viable cells that metabolize the WST-1 reagent. Then the data was analyzed with regression analysis Microsoft Excel 2007 program to determine the concentration with 50% cell viability (50% Cytotoxicity Concentration, CC50). The CC50 values of the fractionated-extract and 70% ethanol extract of J. gendarussa leaves were 94 µg/ml and 78 µg/ml, respectively. The cytotoxicity of fractionated-extract and 70% ethanol extract of J. gendarussa leaves were not significantly different ($p > 0.05$). It can be concluded that the fractionated-extract and 70% ethanol extract of J. gendarussa leaves are not toxic to MOLT-4 cells.

Key words: *cytotoxicity; Justicia gendarussa Burm.f; MOLT-4 cell; WST-1 assay, anti HIV*

ABSTRAK

Justicia gendarussa burm f. (acanthaceae) dikenal untuk aktivitasnya sebagai konstrasepsi pria dan bersifat anti-hiv. Studi ini dirancang untuk mengevaluasi ekstrak J. gendarussa untuk aktivitas sitotoksisitas terhadap sel MOLT-4. Aktivitas sitotoksisitas dari esktrak terfraksinasi dan ekstrak entanol 70% daun J. gendarussa pada sel molt-4 dievaluasi menggunakan sebuah uji WST-1. Sel dengan perlakuan, sel control tanpa perlakuan serta kontrol media juga diuji berulang. Nilai absorbansi diukur pada panjang gelombang 450nm menggunakan microplate absorbance reader (Bio-Rad). Nilai absorbansi rata-rata mengukur formazan yang dihasilkan oleh sel yang bermetabolisis denga reage WST-1. Kemudian data dianalisis menggunakan analisis regresi program Microsoft Excel 2007 untuk menentukan konsentrasi viabilitas sel 50% (50% Cytotoxicity Concentration, CC50). Nilai CC50 dari ekstrak terfraksinasi dan ekstrak entanol 70% daun J. gendarussa adalah 94 µg/ml dan 78 µg/ml, secara berturut-turut. Sitotoksisitas dari ekstrak terfraksinasi dan ekstrak entanol 70% daun J. gendarussa tidak jauh berbeda gendarussa ($p > 0,05$). Dapat disimpulkan bahwa ekstrak terfraksinasi dan ekstrak entanol 70% daun J. gendarussa tidak beracun untuk sel MOLT-4.

Kata kunci: *sitotoksisitas, Justicia gendarussa Burm.f, sel MOLT-4, uji WST-1*

INTRODUCTION

Justicia gendarussa Burm f. (Acanthaceae) leaves are often used in traditional medicine to treat fever, headache, rheumatism, myalgia, respiratory disorders, and back pain.[1] *J. gendarussa* is also used in Papua as a male contraceptive. A pre-clinical study of an alkaloid-free 70% ethanol extract of *J. gendarussa* leaf extract has confirmed male contraceptive activity.[2] The 70% ethanol leaf extract (with alkaloids and without alkaloids) from *J.*

gendarussa also have HIV reverse transcriptase enzyme inhibition activity.[3]

Studies on the *in vitro* and *in vivo* toxicity of *J. gendarussa* leaf extract were previously performed. The administration of a water extract of *J. gendarussa* leaves in male rabbits did not affect liver and renal function.[4] The 60% ethanol and water fraction of the ethanol extract of *J. gendarussa* leaves were non-toxic in acute toxicity and teratogenic tests. Cytotoxicity in human normal lymphocytes cells of the water fraction from the ethanol extract of *J. Gendarussa* leaves had a CC_{50} of 3215.7 µg/ml.[5] Cytotoxicity activities of the methanol extract of *J. Gendarussa* leaves obtained from 4 locations in Malaysia (Regions of Muar, Skundal, Batu Pahat, and Pulai) against human cancer cells lines such as HT-29 (colon adenocarcinoma), HeLa cells (cervix adenocarcinoma), and the BxPC-3 cells (epitheloid cervix adenocarcinoma), as well as MDA-MB-468 and MDA-MB-231 cells (breast cancer cells) using a MTT reagent colorimetric method were also reported as non toxic with CC_{50} values greater than 41 µg/ml. However, the methanol extract of *J. gendarussa* leaves from the Mersing region was toxic to BxPC-3 cells, HeLa cells, MDA-MB-468 and MDA-MB-231 cells with the CC_{50} values of 16 µg/ml, 5 µg/ml, 23 µg/ml, and 40 µg/ml respectively.[6,7]

The leaves of *J. gendarussa* plants contain a substituted aromatic amine,[8] flavonoid glycosides including gendarusin A and B[2], and justidrusamide alkaloids A, B, C, and D.[9] Male contraceptive activity has been attributed to gendarusin A and B isolated from the n-butanol fraction of *J. gendarussa* leaves.[2]

Flavonoids are antioxidants which can protect cells from oxidative stress. Flavonoid compounds from *J. gendarussa* also serve as a natural resource of anti-HIV therapy for AIDS subjects by inhibiting HIV *reverse transcriptase.*[10,11] However, in high concentrations, flavonoids and other polyphenols can also be cytotoxic, causing increased mitochondria permeability, secretion of cytochrome c, capsase activation, increased p53 and p21 levels, depressed bcl-2, apoptosis induction, and cell necrosis.[12, 13, 14, 15,16,17] Alkaloids also have pharmacologic activities useful in the treatment of disease[18] but may also be toxic to man.

Thus, cytotoxicity testing against MOLT-4 cells was performed to evaluate the relative toxicity potential using a fractionated-extract (alkaloid-free) and a 70% ethanol extract of *J. gendarussa* leaves to assess the safety of *J. gendarussa* leaf extracts used in preliminary male contraceptive clinical trials.

MATERIALS AND METHODS

MATERIALS

Plants

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

70% *pharmaceutical grade* ethanol, *pro* HPLC methanol (Merck), sterile water for injection, aquadest (*pure water*) from CRC-EIRD (ITD Surabaya), RPMI-1640 media (Gibco), natrium bicarbonate (Merck), *Fetal Bovine Serum* (FBS) (Gibco) (inactivated at 56 °C for 30 min), Reagent(4-3 (4-iodophenyl)-2-(4-niitrophenyl)-2D-5-tetrazolio]-1,3-benzen disulfonate) (WST-1)(Roche), dimethyl sulfoxide (DMSO) (Sigma), and nitrocellulose 0.2 µm membrane filter (Whatman).

Cells

MOLT-4 cells clone#8 (human T lymphocytes cancer cells line) were obtained from the Bio-safety Level-3 facility CRC-ERID, ITD, Surabaya. MOLT-4 cells were cultured on RPMI-1640 media, with 10% FBS and kept in CCF T_{25} at a temperature of 37 °C in a 5% CO_2 incubator (Sanyo).

METHODS

Preparation of sample

J. gendarussa leaves powder was divided into 2 fractions, a leaf powder with releasing alkaloids and a leaf powder with non-releasing alkaloids. Both powders were extracted using 70% ethanol during 324 hours in macerator and the filtrate obtained evaporated using a rotary evaporator (Buchi). The two extracts were dried at 50 °C until fractionated with 70% ethanol extract (alkaloid-free; 2.4% w/w) and 70% ethanol extract (10.8% w/w).

A stock solution was made by dissolving 100 mg of each extract in 1000 µl DMSO and then diluted using RPMI-1640 medium with 10% FBS to obtain various concentrations (7.8; 15.6; 31.3; 62.5; 125.0; 250.0; 500.0; and 1000.0 µg/ml) for each trial extract. The concentration of DMSO used was less than 1% which does not affect viability.[19]

Detection of Flavonoid in J. gendarussa Leaf Extract

The content of gendarusin A, the major flavonoid in *J. Gendarussa* leaves, was analysed by a Waters HPLC (Agilent 1100, *reverse phase* NovaPack® column C-18 3.9150 mm using a water:methanol (30:70) eluent with a flow rate of 1 ml/min, and a UV detector at 254 nm wavelength).

Cytotoxicity Assay

Cytotoxicity of the extracts on MOLT-4 cells was measured using a colorimetric method with WST-1 reagent (Roche). Briefly, 50 µl MOLT-4 cells (110^5cells/well) were plated in each well on a 96-well microplate. 50 µl of extract at various concentrations were also added to each well, and incubated for 72 hr at 37 °C in a 5% CO_2 incubator. The treatment cells, control cells without treatment and control media were also tested in duplicate. Total volume in each *well* was 100 µl. After 72 hr of incubation, 10 µl WST-1 reagents was added into each well and incubated

for an additional 2 hrs at 37 °C in a 5% CO_2 incubator. The absorbance was measured at a wavelength of 450 nm using a *microplate absorbance reader* (Bio-Rad). The average absorbance measures formazan produced by viable cells that metabolize the WST-1 reagent. The percentage cell viability was determined by the equation below:

$$\text{Cell viability (\%)} = \frac{\text{treatment absorbance} - \text{control media absorbance}}{\text{control cells absorbance} - \text{control media absorbance}} \quad (1)$$

Statistical Analysis

The collected data was analyzed with regression analysis Microsoft Excel 2007 program to determine the concentration with 50% cell viability (50% Cytotoxicity Concentration, CC_{50}). The comparison of cytotoxicity activities of both extracts were tested using a paired t-test analysis (Microsoft Excel 2007 program). The difference was considered to be significant if the probability was $p < 0.05$.

RESULTS AND DISCUSSION

The leaf extracts of *J. gendarussa* tested had low toxicity to MOLT-4 cells with decreased MOLT-4 cell viability with increasing extract concentrations (Table 1). Based on regression analysis, the CC_{50} of the 70% ethanol extract was 78 µg/ml, while fractionated-70% ethanol extract was 94 µg/m.

Table 1. Cytotoxicity test of 70% ethanol extract and fractionated-70% ethanol extract to MOLT-4 cells incubated for 72 hr

Extract Concentration (µg/ml)	Cells Viabilities (%) ± SD	
	70% Ethanol Extract	Fractionated-70% Ethanol Extract
7.8	101.4 ± 2.9	100.4 ± 1.2
15.6	91.3 ± 4.9	89.8 ± 3.8
31.3	72.2 ± 8.8	81.1 ± 12.1
62.5	51.2 ± 1.3	67.7 ± 6.1
125.0	35.1 ± 6.1	37.4 ± 2.5
250.0	15.1 ± 6.1	22.5 ± 0.6
500.0	8.9 ± 5.6	7.3 ± 0.9
1000.0	3.2 ± 11.8	4.7 ± 0.9

A comparison of extract cytotoxicity activity was performed using a paired t-test analysis based on the percentage of cell viability for each treatment. Based on the t-test, t = -1.786, t table $_{(0.05)}$ = ± 2.365, the significance value is 0.117 or 11.7% which is larger than 0.05 or 5%. Based on t-test results, there was no significant difference for cytotoxicity activitiy on MOLT-4 cells between the fractionated-70% ethanol extract and the 70% ethanol extract of *J. gendarussa* leaves ($p > 0.05$).

HPLC chromatograms show gendarusin A content in the fractionated-70% ethanol extract (Fig. 1b) and the 70% ethanol extract (Fig. 1c) as a major flavonoid component of *J. gendarussa* leaves. Fractionated-70% ethanol extract and 70% ethanol extract of *J. gendarussa* leaves contain 0.53% and 0.95% of gendarusin A, respectively.

Cytotoxicity activity were evaluated to identify the relative toxicity of fractionated-70% ethanol extract and 70% ethanol extracts from *J. Gendarussa* leaves to MOLT-4 human T-lymphocytes line cells using a WST-1 test. The test is based on the reduction of tetrazolium sodium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzendisulfonate) (*water-soluble tetrazolium salt*, WST-1) by a succinate-tetrazolium reductase system of the mitochondria respiratory chain. This enzyme system is only active in viable cells. The WST-1 reduction process produces soluble formazan with a bright colour. Absorbance measurements of formazan are directly related to cell viability.[20,21]

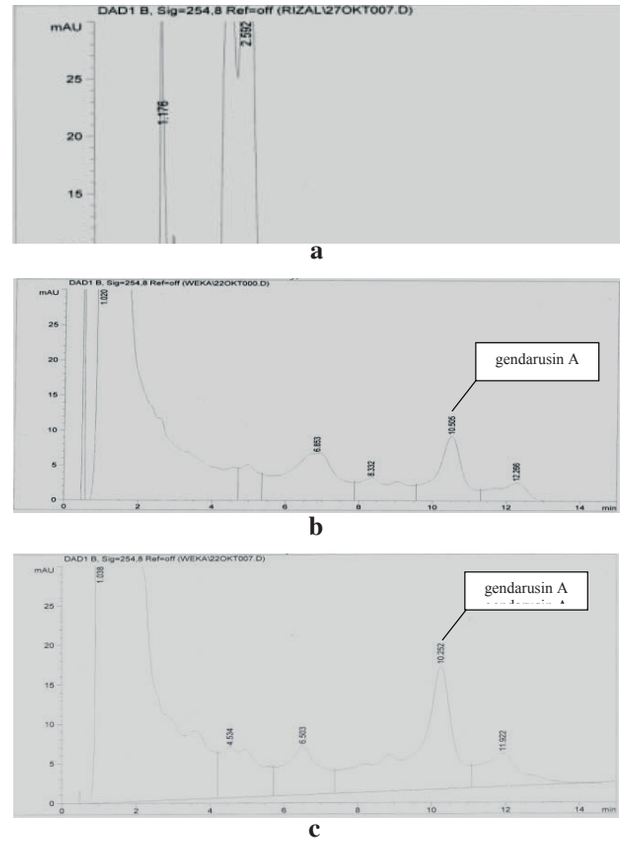

Figure 1. Chromatogram profile of (a) gendarusin A - 12.8 µg/ml solution; (b) gendarusin A in 5000.0 µg/ml of the fractionated-70% ethanol extract of *J. gendarussa* leaves; and (c) gendarusin A in 5000.0 µg/ml of the 70% ethanol extract of *J. gendarussa* leaves.

The cytotoxicity activity of the extract was measured as the concentration of extract that reduces cell viability or cell growth by 50% (CC_{50}). The cytotoxicity levels were based on previous studies where CC_{50} values less than 20 µg/ml were considered as cytotoxic, 21–40 µg/ml as low cytotoxicity, and over 41 µg/ml as not cytotoxic.[22,23,24]

Using this criteria, fractionated-70% ethanol extract (CC_{50} -93 µg/ml) and 70% ethanol extract (CC_{50}-78 µg/ml) of *J. gendarussa* leaves are considered non-cytotoxic to MOLT-4 cells.

The cytotoxicity activity of fractionated-70% ethanol extract and 70% ethanol extract of *J. gendarussa* leaves at various concentrations on MOLT-4 cell viability are found in Fig 2. The reduction of cell viability is reflected by the inhibition of cell growth related to the suppression of cell proliferation activities so that the total number of dividing or living cells are decreased. Various cell signalling activities involved in protein expression of the programmed cells death such as *bid, bax*, and *bcl*-2 are likely to be activated when cell lines are exposed to active compounds contained in the extract[25] causing an increase in programmed cell death (apoptosis).[26] Testing by using tetrazolium only measures the formation of formazan (which is related to mitochondria living cell activities) but it is not able to determine the cause of cell death.[27]

There was no statistical difference between the two extracts despite having twice the amount of gendarusin A in the extract which indicates that gendarusin A probably does not significantly contribute to the cytotoxicity of the extract. The alkaloids present in the 70% ethanol extract probably contributed to the cytotoxicity but also did not result in a significant difference between the alkaloid free and the alkaloid containing extracts.

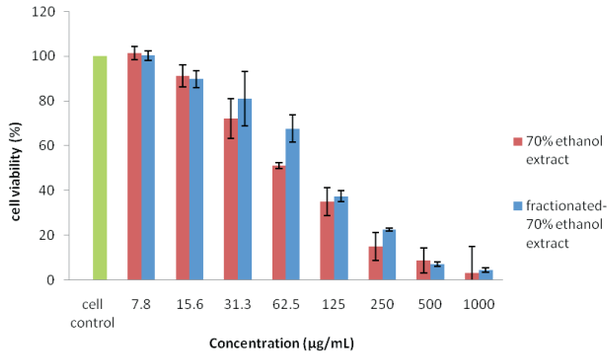

Figure 2. Comparison of the effect of various concentrations of fractionated-70% ethanol extract and 70% ethanol extract of *J. gendarussa* leaves on MOLT-4 cell viability with a 72 hr incubation period.

CONSLUSIONS

Fractionated-70% ethanol extract and 70% ethanol extract of *J. gendarussa* leaves are relatively non-cytotoxic to MOLT-4 cells with no significant difference of cytotoxicity between the fractionated-70% ethanol extract and 70% ethanol extract ($p > 0.05$).

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