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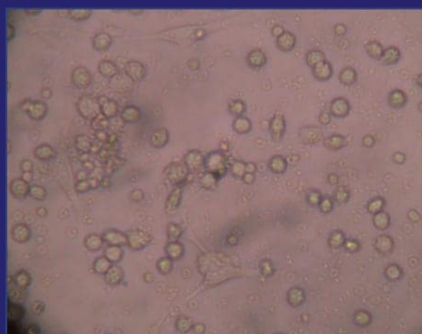

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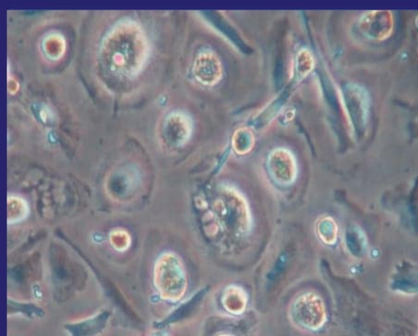

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Indonesian Journal of
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Research Report

EFFECT OF FREE ALKALOID AND NON-FREE ALKALOID ETHANOL 70% EXTRACT OF *JUSTICIA GENDARUSSA* BURM F. LEAVES AGAINST REVERSE TRANSCRIPTASE HIV ENZYME IN VITRO AND CHEMICAL COMPOUND ANALYSIS

Bambang Prajogo[1], Prihartini Widiyanti[2,3], Hafrizal Riza[4]
[1] Department of Pharmacognosy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia
[2] Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia
[3] Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, East Java, Indonesia
[4] Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Tanjungpura, Pontianak, West Kalimantan, Indonesia
Corresponding author: prajogo_ew@yahoo.com

ABSTRACT

HIV-AIDS is a global problem and the deadliest disease in the world. One of HIV and AIDS prevention strategy can be done with traditional medicine research program from natural resource that has anti-HIV AIDS activity. It has been found that 70% ethanol extract of Justicia gendarussa Burm.f leaves, alkaloid free and alkaloid non-free, has a strong inhibitory activity against HIV reverse transcriptase enzyme, as an effort to find a solution in the face of HIV AIDS prevalence that is still high with problem of HIV-AIDS treatment such as side effects and resistances. Justicia gendarussa had already known for having an effect anti-HIV and therefore we were looking at the mechanism of inhibition of HIV Reverse Transcriptase enzyme. Both types of extracts were tested in vitro using ELISA technique and analysed chemical content of Gendarusin A as anti-HIV using high performance liquid chromatography. ELISA test results obtained percent inhibition, respectively for 254.2, 254.2, 235.6, and 279.7 for the concentration of 5 ppm, 10 ppm, 15 ppm and 20 ppm of free alkaloid extract and 169.0, 164.0, 130.5 and 369.5 for the concentration of 5 ppm, 10 ppm, 15 ppm and 20 ppm of non-free-alkaloid extract. The results of high performance liquid chromatography obtained Gendarusin A in the free-alkaloid extract at retention time 8.402 minutes and non-free alkaloid extract at retention time 8.381. Therefore, these results concluded that the Justicia gendarussa Burm.f can be a useful resource for the isolation and development of new anti-HIV.

Key words: *Justicia gendarussa; 70% ethanol extract; free and non-free alkaloid; reverse transcriptase; anti-HIV*

ABSTRAK

HIV-AIDS merupakan permasalahan global dan penyakit yang mematikan di dunia. Salah satu strategi pencegahan HIV-AIDS dapat dilakukan dengan program penelitian pengobatan tradisional dari sumber daya alam yang memiliki aktivitas anti-HIV AIDS. Telah ditemukan bahwa ekstrak ethanol 70% daun Justicia burm.f gendarussa, bebas alkaloid dan mengandung alkaloid, memiliki aktivitas inhibitor yang kuat terhadap enzim HIV reverse transcriptase, sebagai upaya dalam rangka mencari solusi menghadapi prevalensi HIV AIDS yang masih tinggi dengan masalah pengobatan HIV-AIDS seperti efek samping dan resistansi. Justicia gendarussa telah diketahui memiliki efek anti-HIV dan perlu diketahui mekanisme penghambatan enzim HIV reverse transcriptase. Kedua jenis ekstrak diuji in vitro menggunakan teknik ELISA dan dianalisis kandungan kimia Gendarusin A sebagai anti-HIV menggunakan High Performance Liquid Chromatography (HPLC). Hasil tes ELISA diperoleh persen inhibisi, masing-masing untuk 254.2, 254.2, dan 279.7, 235.6 untuk konsentrasi 5 ppm, 10 ppm, 15 ppm, dan 20 ppm ekstrak bebas alkaloid dan 169.0 bebas, 164.0, 369.5, 130.5 untuk konsentrasi 5 ppm, 10 ppm, 15 ppm, dan 20 ppm dari ekstrak yang mengandung alkaloid. Hasil High Performance Liquid Chromatography (HPLC) menunjukkan Gendarusin A pada ekstrak bebas alkaloid pada waktu retensi 8.402 menit dan ekstrak yang

mengandung alkaloid pada waktu retensi 8.381. Hasil tersebut mengarah pada kesimpulan bahwa Justicia gendarussa burm.f dapat berpotensi bagi isolasi dan pengembangan anti-HIV baru.

Kata kunci: *Justicia gendarussa; ekstrak ehanol 70%; bebas dan tidak bebas alkaloid; anti-HIV*

INTRODUCTION

HIV-AIDS is a global problem and the deadliest disease in the world. According to WHO global report, the number of AIDS deaths in the world in 2009 reached 1.8 million people[1]. Whereas based on the Health Ministry data, although the total HIV and AIDS cases nationwide declined from 2011 as many as 21.031 and 4162 into 9.883 and 2.224 in 2012, but it is still relatively high.[2] Seen from a treatment, medical efforts of the HIV-AIDS treatment service still face some problem. For example, the antiretroviral utilization which is the dose and side effect are very limited. Toxicity and side effects affecting patient obedience to antiretroviral. Furthermore, antiretroviral utilization this time are resistant that cause the failure of therapy.[3]

One of HIV and AIDS prevention strategy can be done with traditional medicine research program from natural resource that has anti-HIV AIDS activity. Traditional medicine research is directed to find a scientific evidence on it.[4] Natural resources still have an important role as an initial material invention of new drugs.[5] Based on a published report in 2007, there were 974 molecular compound that 63% of them come from natural or semisynthetic derivatives from natural materials.[6]

The reverse transcriptase enzyme has an important role in the life cycle of HIV because reverse transcription is an early phase of viral replication in the cell host. All the proteins and enzymes that play an important role in the new virus formation are not carried by the virus but using enzymes and proteins in host cells.[7] Furthermore, the reverse transcriptase enzyme together with an integrated enzyme is derived from a virus that enters the host cell in fusion phase.[8] Therefore, the drugs development that act on the reverse transcriptase enzyme will inhibit the next cycle process directly, starting from reverse transcription of the virus RNA into DNA, the integration of DNA virus on host cell DNA, and core replication to the virus proteins formation. Inhibition on phase after transcription is still possible to set an infection in the host cell because the virus DNA can settle along with the host cell DNA. Therefore, inhibition of the enzyme reverse transcriptase can reduce a HIV infection.[7]

Currently, it is being developed an anti-HIV drugs derived from natural medicine, namely *Justicia gendarussa* Burm.f. Research has been done on them to test the effect of hexane, methanol and ethanol of *Justicia gendarussa* Burm.f drug against HIV virus in vitro methanol and ethanol extracts obtained 70% alkaloid-free give a decrease in the amount of virus results.[9]

The part of *Justicia gendarussa* Burm.f herb showed inhibitory activity analog reverse transcriptase enzyme substrates in vitro.[10] Isolate of the pure compound flavonoid apigenin showed inhibition of HIV reverse transcriptase enzyme activity as a substrate analog and HIV protease enzyme in vitro.[11] The main content of 70% ethanol extract *Justicia gendarussa* Burm.f is apigenin. A few compounds either major or minor component can give a synergistic effect as an anti-HIV through the same or different mechanism.[12] Therefore, this study aimed to test the inhibitory activity of the Reverse Transcriptase HIV enzyme using 70% ethanol extract free and non-free alkaloid.

MATERIALS AND METHODS

Materials

*Justiciagendarussa*Burm.f leaf obtained from cultivated plants in Trawas, Mojokerto, East Java. Roche RT Activity Kit obtained from PT. Roche, Germany. Materials to extract, alkaloid test and chemical content are 70% ethanol, dichloromethane, methanol, hexane, distilled water, citric acid, filter paper, dragendorf reagent, Silica gel GF 254.Extraction tool set such as macerator extraction, Memmert oven, evaporator buchi, Julabo USR 3 ultrasonic, iMark Microplate Absorbance Reader, HPLC tool set: Agilent1100, reversephase column C18 Nova-pak® sized 3,9 × 150 mm.

Research Place

Research had been done in two laboratories, Laboratory Pharmacognosy Faculty of Pharmacy, Airlangga University and Institute of Tropical Disease laboratory, Airlangga University.

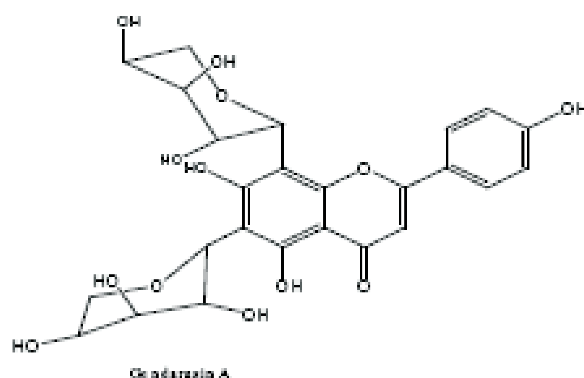

Figure 1. Gendarusin A Structure. (Prajogo BEW, 2010)

Methods

Simplicia of *Justicia gendarussa* Burm.f leaves were divided into two sample groups. First group acidified to release the alkaloids and the second group did not to acidified. Both two sample groups were macerated with 70% ethanol and then concentrated. To ensure of free alkaloid, the first group tested the alkaloid free using Thin Layer Chromatography with stationary phase Silica Gel GF 254 and the mobile phase dichloromethane: methanol, 9:1 with the spray Dragendorf reagent. Both of samples tested its activity in an enzyme activity inhibition Reverse Transcriptase HIV using Elisa and determined the type of inhibitor. Both samples were also analysed to determine levels of chemical content Gendarusin A based on previous research that isolates apigenin has Reverse transcriptase HIV inhibitory activity. The conditions that used in HPLC was methanol eluent: water (30:70), flow 1 ml/min, stop time 25 minutes, a wavelength of 254 nm.

RESULTS AND DISCUSSION

In this study, it had been tested to know the inhibitory activity of *Justicia gendarussa* Burm.f 70% ethanol extract against Reverse Transcriptase HIV enzyme and acidification differences treatment to know the effect of alkaloid on the inhibition of the enzyme Reverse Transcriptase HIV. Extraction was done by ethanol 70% maceration because the previous studies showed the extract can decrease the amount of HIV virus in vitro culture.

The first group was tested to ensure that it was free from alkaloid as shown in Figure 2. The stain A that was free from alkaloid extract was not showed red-orange stain like stain B and C.

Inhibition testing of Reverse Transcriptase HIV enzyme was obtained by using the formula (1).

$$\%Inhibition = \left[\frac{A_0 - A_1}{A_0}\right] \times 100\% \quad (1)$$

Explanation:
A_0: blank absorbance
A_1: sample absorbance

Table 1 shows the both extracts had inhibitory activity of the Reverse Transcriptase HIV enzyme that was indicated by the percent inhibition.

Table 1. Inhibition percentage of 70% ethanol extract alkaloid-free and non-free of *Justicia gendarussa* Burm.f leaves to the activity of the Reverse Transcriptase HIV enzyme

Sample	Concentration	% Inhibition
A	20 ppm	279,7
	15 ppm	235,6
	10 ppm	254,2
	5 ppm	254,2
B	20 ppm	369,5
	15 ppm	130,5
	10 ppm	164,0
	5 ppm	169,0
K+	100 µg/ml	83
K-	-	-

Explanation:
A : Non-free alkaloid extract
B : Free alkaloid extract
K^+ : Positive control (Doksorubisin 100 µg/ml)
K^- : negative control (tested solution without enzyme and sample)

For the chemical substitute analysis of Gendarusin A obtained at retention time 8.402 minutes for the alkaloid-free extract and retention time 8, 381 minutes in Fig 3 compared with standard Gendarusin A 9.6 ppm with a retention time of 8.590 minutes as shown in Fig 2.

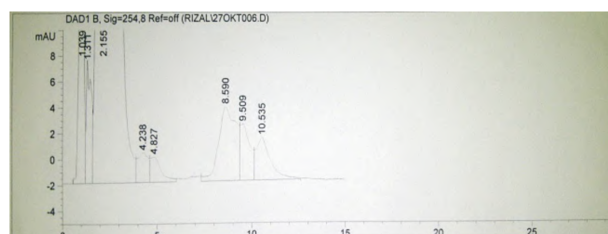

Figure 2. Gendarusin A 9,6 ppm standard chromatogram

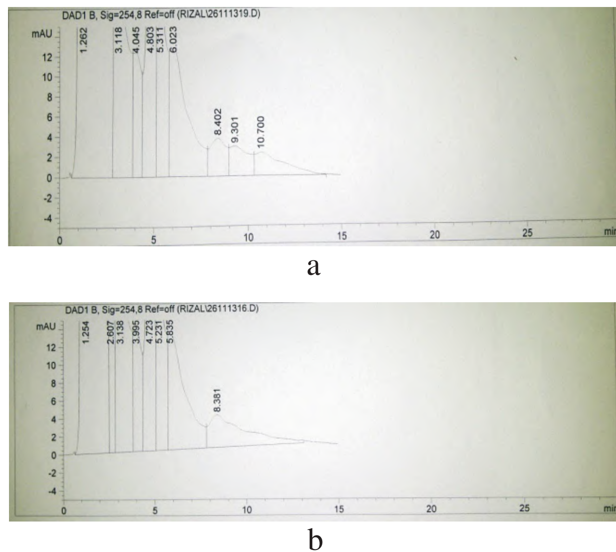

Figure 3. Ethanol 70% Extract of *Justicia gendarussa* Burm.f, Leaves Chromatogram (a) alkaloid-free and (b) non alkaloid-free.

CONCLUSIONS

From these results, it can be concluded that the 70% ethanol extract of *Justicia gendarussa* Burm.f leaves 60th alkaloid- free and non-alkaloid-free have inhibitory activity of the Reverse Transcriptase HIV enzyme.

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Literature Review

SYNTHESIS OF METAL-ORGANIC (COMPLEXES) COMPOUNDS COPPER(II)-IMIDAZOLE FOR ANTIVIRAL HIV CANDIDATE

Teguh Hari Sucipto[1,2], Fahimah Martak[2]
[1]Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.
[2]Natural Product Compound and Synthesis Laboratorium, Departement of Chemistry, Mathematics and Natural Science Faculty, Sepuluh Nopember Institute of Technology
Corresponding author : teguhharisucipto@gmail.com

ABSTRACT

The human immunodeficiency virus (HIV) is viruses known as rotaviruses. Potential target for therapeutic is reverse transcriptase (RT), possesses an RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and ribonuclease H fuctions. Imidazoles have high anti-HIV inhibitory activity, some derivates of imidazole reported drugs. 8-chloro-2,3-dihydroimidazole[1,2-b][1,4,2]benzodithi-azine-5,5-dioxides and 9-chloro-2,3,4-trihydropyri-mido[1,2-b][1,4,2]benzodithi-azine-6,6-dioxides. This compounds succesfully identified anti-HIV activity. Copper is a bio-essential element and copper complexes have been extensively utilized in metal mediated DNA cleavage for the generation of activated oxygen species. It has been reported that teraaza macrocyclic copper coordination compounds have anti-HIV activities. Studies have shown that these macrocyclic complexes can react with DNA in different binding fashions and exhibit effective nuclease activities. Complex compounds are compounds in which there is an atom that acts as the central atom and trotter group of molecules that can be either neutral or charged ions. Application a metal-organic (complex) compounds, especially copper metal and derivates of imidazole. So, in this study can explore new anti-HIV candidate.

Key words: *Complexes compound, copper, imidazole, antiviral, HIV*

ABSTRAK

Human immunodeficiency virus (HIV) adalah virus yang termasuk golongan rotavirus. Target potensial untuk terapi adalah reverse transcriptase (RT), memiliki sebuah DNA-dependent RNA polimerase, DNA-dependent DNA polimerase dan ribonuklease. Imidazol memiliki aktivitas penghambatan anti-HIV yang tinggi, beberapa turunan dari imidazol melaporkan obat. 8-kloro-2,3-dihydroimidazole [1,2-b] [1,4,2] benzodithi-azine-5,5-dioksida dan 9-chloro-2,3,4-trihydropyri-mido [1,2-b] [1,4,2] benzodithi-azine-6,6-dioksida. Ini senyawa aktivitas anti-HIV berhasil diidentifikasi. Tembaga adalah unsur dan tembaga kompleks bio-penting telah banyak digunakan dalam logam dimediasi pembelahan DNA untuk generasi spesies oksigen aktif. Telah dilaporkan bahwa senyawa koordinasi tembaga teraaza makrosiklik memiliki kegiatan anti-HIV. Penelitian telah menunjukkan bahwa kompleks makrosiklik dapat bereaksi dengan DNA di mode mengikat yang berbeda dan menunjukkan aktivitas nuklease yang sangat efektif. Senyawa kompleks adalah senyawa yang ada atom yang bertindak sebagai atom dan dikelilingi oleh molekul yang dapat berupa ion netral atau ion pengganti. Aplikasi logam-organik (kompleks) senyawa, terutama logam tembaga dan turunan dari imidazol. Jadi, pada studi ini dapat dipelajari kandidat anti-HIV baru.

Kata kunci: *Senyawa kompleks, tembaga, imidazole, antivirus, HIV*

INTRODUCTION

The human immunodeficiency virus is a number of class of viruses known as rotaviruses, was identifiend as the causative agent in the transmission and development of acquired immune deficiency syndrome (AIDS). The replicative cycle of HIV provides many potential targets for therapeutic intervation. Reverse transcriptase

(RT), possesses an RNA-dependent DNA polymerase, a DNA dependent DNA polymerase and ribonuclease H fuctions.[1]

Imidazoles have high anti-HIV inhibitory activity[2], some derivates of imidazole reported drugs. Imidazole a ring substituted and pirimidine ring for potent inhibitory activity against RT. These cmpund showed minimal cytotoxicity and are therefore suitable for antiviral development.

Complex compounds are compounds in which there is an atom that acts as the central atom and trotter group of molecules that can be either neutral or charged ions. This trotter group called ligands. Complex compounds formed are influenced by the nature of the ligand, which includes the alkalinity, bond, and chelate effects.

Copper is a bio-essential element and copper complexes have been extensively utilized in metal mediated DNA cleavage for the generation of activated oxygen species. It has been reported that tetraaza macrocyclic copper coordination compounds have anti-HIV activities.

This papers reviews about imidazole potency and copper for anti-HIV. So, in this study can explore drug from the mixture compound, metal-organic compound, especially Cu-imidazole complexes.

Imidazole Compound and Derivates

Brzozowski et al., (2006) prepared new compound with modifications on the imidazole [2]. We present the synthesis 8-chloro-2,3-dihydroimidazole[1,2b][1,4,2]

benzodithi-azine-5,5-dioxides and 9-chloro-2,3,4-trihydropyrimido[1,2b][1,4,2]

benzodithi-azine-6,6-dioxides (figure 1). Succesfully identified anti-HIV activity ECO_{50} 0.09 µM. This compounds showed minimal cytotoxicity and suitable for antiviral development. In the compounds, methyl group at position 7 showed the highest anti-HIV activity cause electron-donating. Compounds showed significant cytotoxicity in cell-based assays even though they were very effective in HIV-1 integrase-based assays.[2]

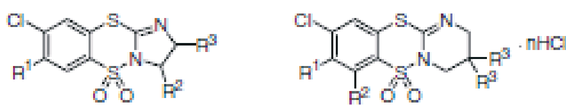
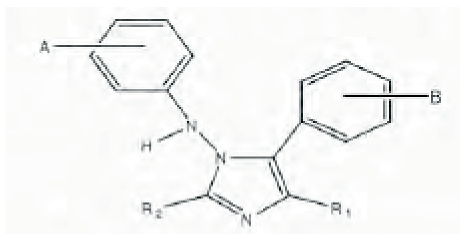


Figure 1. Modification of imidazole.[2]



Figures 2. 5-phenyl-1-phenylamino imidazole.[3]

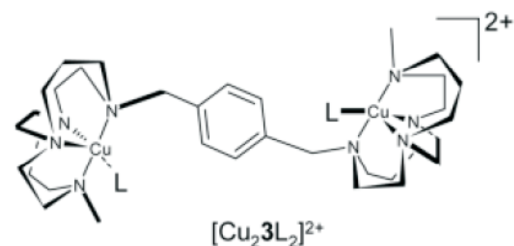
Anti-HIV of 5-phenyl-1-phenylamino-imidazole have been cytotoxicity data in QSAR study. In the QSAR study, imidazole derivate presence of hydrogen bond donor groups appears to be an important feature for reducing the cytotoxicity. Molecular size can also important for determining the cytotoxicity.[3]

In 2004, 1-[2-(alkylthio-1-imidazolyl)carbonyl]-4-[3-(isopropyl amino)-2-pydridyl] piperazines, the compound were tested for anti-HIV activity and had maximum precent of protection $2x10^{-5}$M.[1]

2-alkylthio-1-[4-(1-benzyl-2-athyl-4-nitro-1H-imidazole-5-yl)-piperazin-1-yl] ethanones and alkyl-[4-(1-benzyl-2-ethyl-4-nitro-1H-imidazol-5-yl)-piperazin-1yl) ketones, the newly synthesized compounds were assayed against HIV-1 and HIV-2 in MT-4 cells. The compounds were showed inhibition of HIV-1 (EC_{50} 0.45 µg mL^{-1}) and HIV-2 (0.50 µg mL^{-1}). The target is non-nucleoside reverse transcriptase inhibitor.[4]

Copper For Antiviral HIV Activity

Copper is a bio-essential element and copper complexes have been extensively utilized in metal-mediated DNA cleavage for the generation of activated oxygen species. It has been reported that teraaza macrocyclic copper coordination compounds have anti-HIV activities. Studies have shown that these macrocyclic complexes can react with DNA in different binding fashions and exhibit effective nuclease activities.[5]

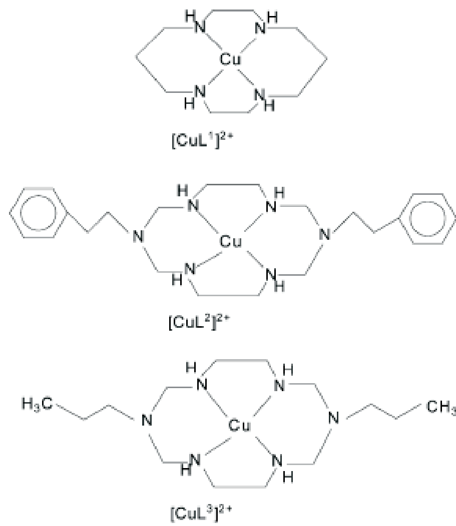


Figures 3. Macrocyclic copper(II) complexes.[5]

At 2010, copper(II) containing bis-macrocyclic $[Cu_2 3L_2]^{2+}$ has improved anti-HIV potency in vitro (EC_{50} 4.3 nM). The interaction of the metallodrug has been optimized by using ultra rigid chelator units that offer an equatorial site for coordination to the amino acid side chains of the protein.[6]

Cu_2-xylyl-bicyclam also exhibits anti-HIV activity. It was used Cu^{2+}-cyclam as a paramagnetic probe to investigate interactions of metal-locyclams with the model protein target in solution.[7]

Copper complexes were substrated competitive inhibitors for HIV-1 protease. For example, [bis-(2-pyridylcarbonyl)-amido] copper(II) nitrate dihydrate binds with an inhibiton constant of 480 µM. molecular modeling suggests that the catalytic water between Asp25 and Asp125 of HIV-1 protease is directly coordinated to the Cu(II) ion.[8]

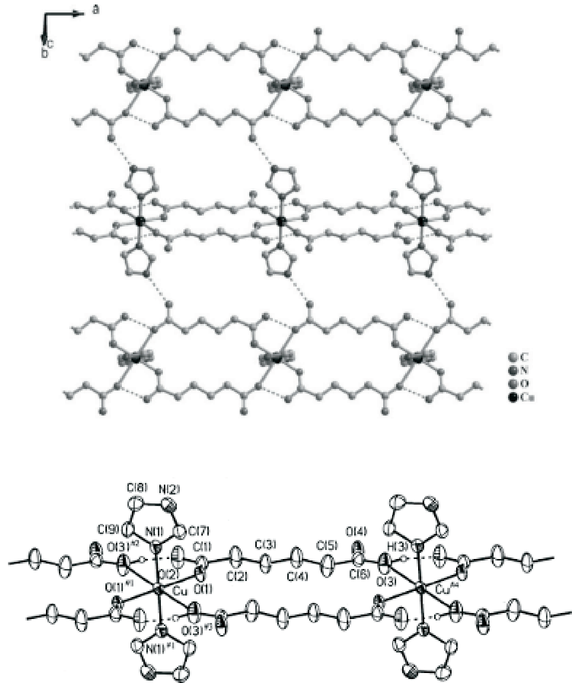


Figures 4. Copper(II) bis-macrocyclic.[8]

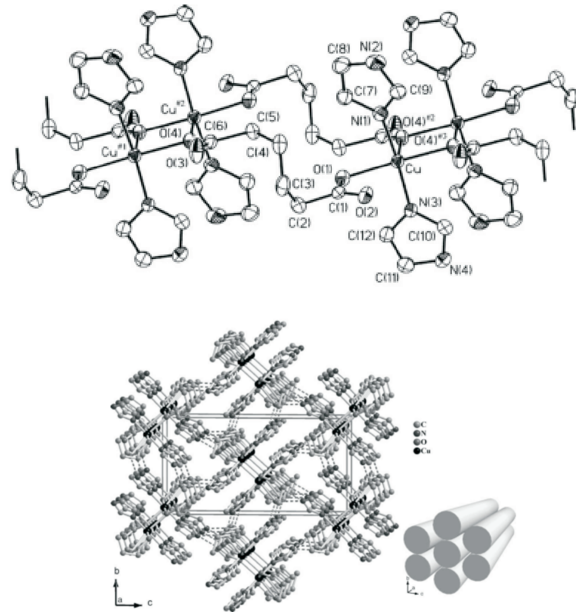
Complex Compounds of Cu(II)-Imidazole

Complex compounds copper(II) with monodentate ligand 1 imidazoles

Ying et al., Synthesis complex compounds of Cu(II) as the central atom with ligands that have a monodentate imidazole strutur octahedral geometry. Complex compound formed is $Cu(C_3N_2H_4)_2(HL)_2$ and $Cu(C_3N_2H_4)_{2L}$ with $C_3N_2H_4$ is an imidazole and HL is adipic acid. In the structure of the complex compounds occur hydrogen bonds between the NH---O into a compound supermolecule due to polymerization. In the complex compound $Cu(C_3N_2H_4)_{2L}$, Cu atom has five coordination centers of CuN_2O_3 pyramidal, then bind to ligands bridge ligand monodentate adipic acid so as to form a polymerization.[9]

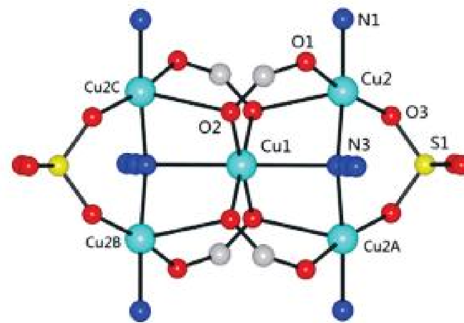


Figures 5. (a) Complex compound $Cu(C_3N_2H_4)_2(HL)_2$, (b) Hydrogen bonding structure.[9]

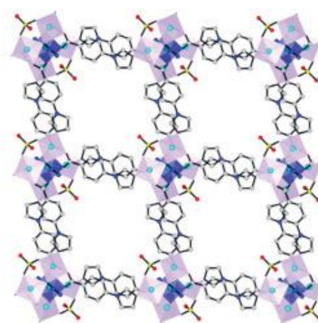


Figures 6. (a) Complex compound $Cu(C_3N_2H_4)_2L$, (b) Hydrogen bonding structure.[9]

In the year 2011 has been synthesized $[Cu_{5(}IBA)_{4(}N_3)_{2(}SO_4)_2]$.$4H_2O$ with HIBA is 4- (imidazol-1-yl) benzoate -acid by Liu. This complex compound has a symmetrical structure with an angle {Cu2, Cu2A, Cu2B, Cu2C} is 71.57° and 108.43°. Magnetic properties of complex compounds are ferromagnetic.[10]

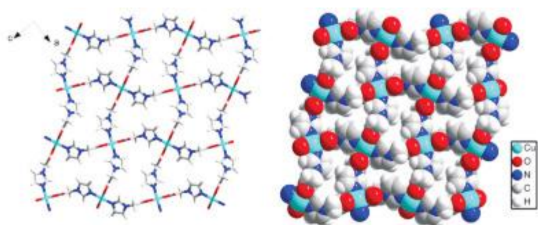


Figures 7. Complex compound $[Cu_5(IBA)_4(N_3)_2(SO_4)_2]$.$4H_2O$.[10]



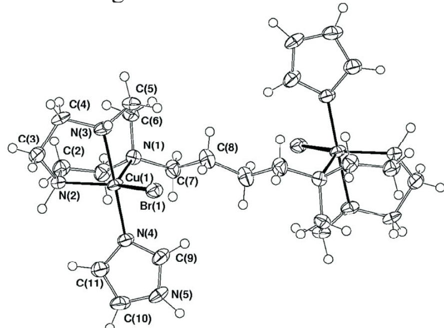
Figures 8. 2D structure $[Cu_5(IBA)_4(N_3)_2(SO_4)_2]$.$4H_2O$ of hydrogen bonding effect. [10]

By Li et al., In 2013 have synthesized a complex compound used for heterogeneous catalysts. This is a complex compound $[Cu(IMA)_2]_n$, synthesized in methanol and ambient temperature. The catalytic properties of complex compounds is very good because it has the results (%) is high and the higher the stability of the compound.[11]



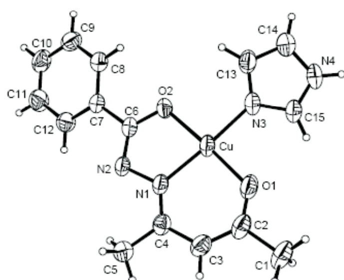
Figures 9. 2D Crystal structure of MOF with 4 coordination Cu^{2+}.[11]

$[Cu_2L^{but(}imidazole)_{2Br2}](ClO_4)_2$ have been successfully synthesized by Graham et al., In 2005 ago. The molecular structure above has cation $[Cu_2L^{but(}imidazole)_{2\ Br2}]^{2+}$ and perchlorate anions. L^{but} ligand is anticonformation so as to cause the complex compounds into centrosymmetry with bridge butane, Cu---Cu 8446 Å. Copper (II) as a coordination center, coordinating with the anions bromide dn monodentate ligand N-imidazole.[12]



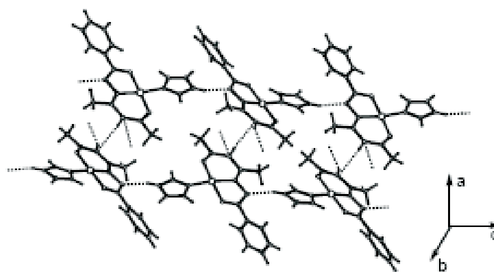
Figures 10. $[Cu_2L^{but}(imidazole)_2Br_2]\ (ClO_4)_2$ Compound.[12]

Complex compounds [Cu(bhac)(Himdz)] has a central atom Cu(II) as a coordination center, acetylacetone benzoilhidrazona tridentat ligand and monodentate ligands imidazole. These compounds can be formed due to metal ion coordination with enolate-O, imine-N and the deprotonated amide-O atom sixth and fifth ring chelate.[13]



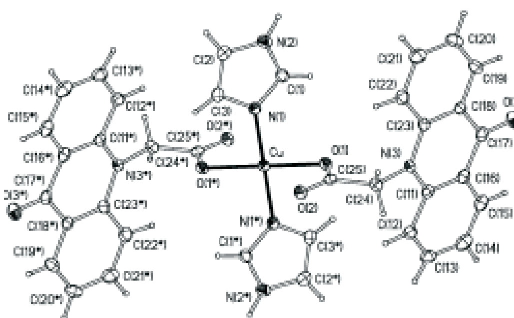
Figures 11. Complex compound [Cu(bhac)(Himdz)].[13]

Then through intramolecular hydrogen bonds NH---N form a crystalline regularity, this is called polymerization. The effective magnetic moment of these compounds is 1.86 μB. Weak antiferromagnetic because their 2-apical equatorial, chloro and bridges asetato.[13]



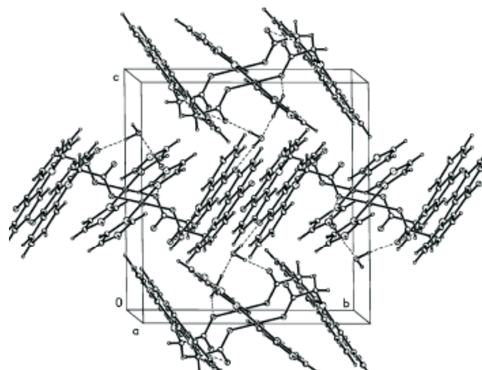
Figures 12. 2D structure of crystal [Cu(bhac)(Himdz)].[13]

Synthesis of bis (9,10-dihydro-9 oxo-10-acrydinacetato) bis (imidazole) Copper (II)tetrahydrate.[14] Monomer crystal structure of $Cu(CMA)_{2(}Him)_2$ will react intermolecular hydrogen bond with water molecules.



Figures 13. Complex compound $Cu(CMA)_2(Him)_2$.[14]

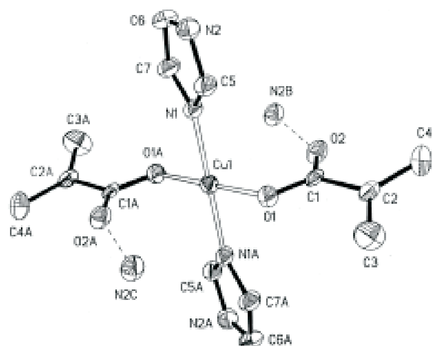
Cu complex compounds $(CMA)_{2(}Him)_2$ can bind hydrogen NH---O form a crystalline order as follows,



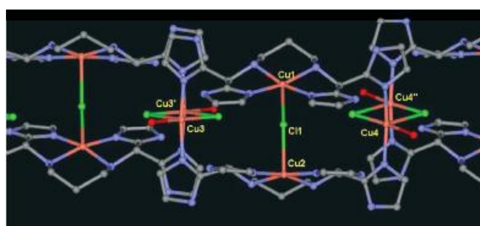
Figures 14. 2D crystal $Cu(CMA)_2(Him)_2$.[14]

In 2005, Song et al., Have managed to synthesize $[Cu_{4(}H_3L)(H_2L)CL_3(H_2O)_2]\ Cl_2.5H_2O$. This complex compound used as a ligand clorate ligands bridge connecting Cu-Cu so that a Cu-Cl-Cu. Furthermore, with

the intramolecular hydrogen bond is formed a polymer complex.[15]

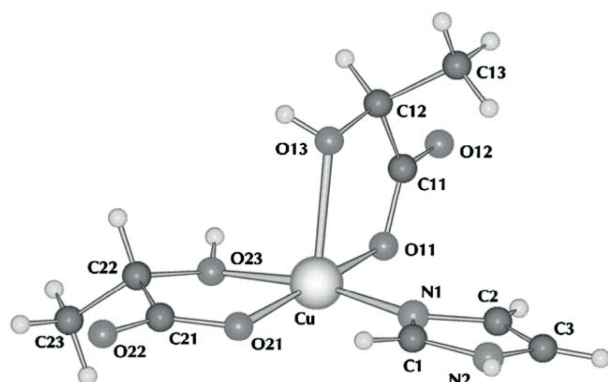


Figures 15. Complex compound $[Cu_4(H_3L)(H_2L)Cl_3(H_2O)_2]Cl_2.5H_2O$. [15]



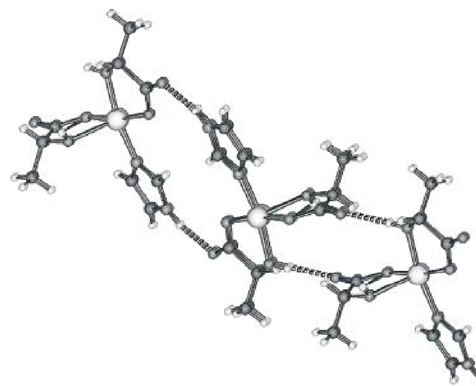
Figures 16. 1D polimer structure of complex compound $[Cu_4(H_3L)(H_2L)Cl_3(H_2O)_2]Cl_2.5H_2O$. [15]

Carbalo et al., 2004 perform synthesis of $[Cu(HL)_2(Im)]$ with the coordination geometry pyramide structure.[16]



Figures 17. Complex compound $[Cu(HL)_2(Im)]$. [16]

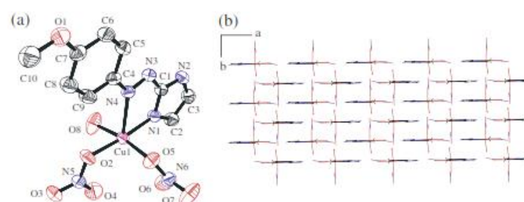
The complex compounds will undergo intramolecular hydrogen bonds form a continuous crystal. The crystals were formed as glasses with Cu(II) as the central atom.



Figures 18. Crystal $[Cu(HL)_2(Im)]$ showed 2D after hydrogen donding. [16]

Complex compounds copper (II) with a bidentate ligand 1 imidazoles

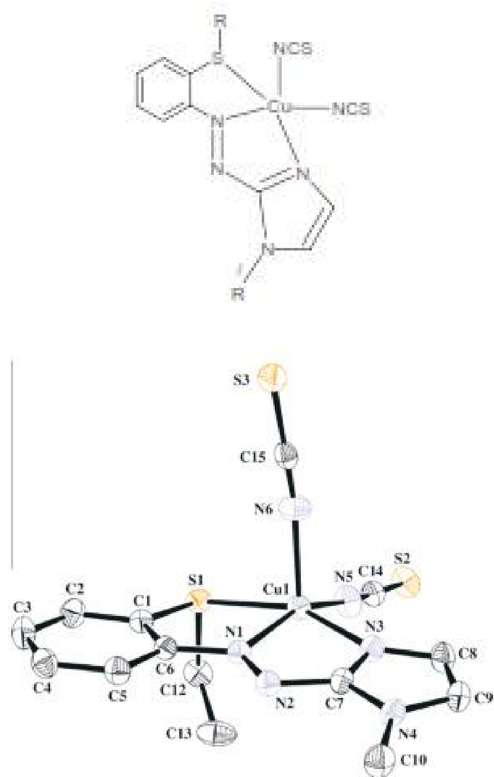
In 2010 Pramanik et al., Have succeeded in synthesizing complex compounds $[Cu(II)(L_2)(H_2O)(NO_3)_2]$ with the central atom Cu(II) which has a planar coordination giometry pyramid, nitrate as a monodentate ligand and two atoms N on imidazole freely donate an electron pair to form a common bond of Cu(II) which is referred to as a bidentate ligand. Furthermore, there is intramolecular hydrogen bond bond, the N atom of the imidazole with oxygen atoms from nitrate. So as to form a polymerization, namely a crystal.[17]



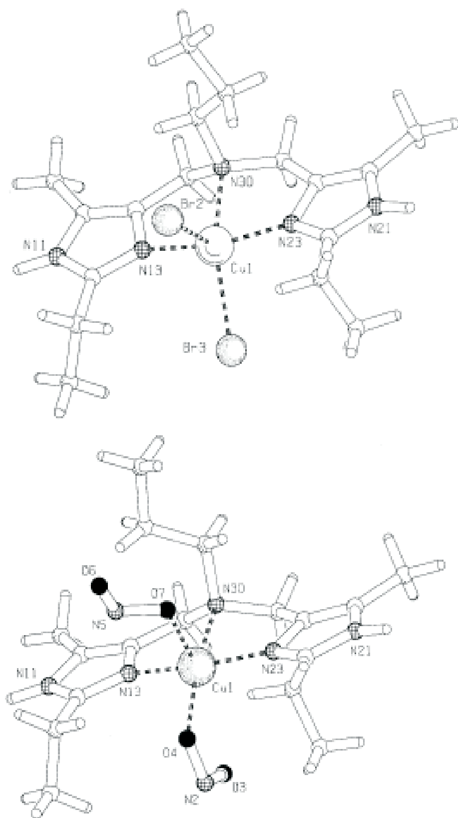
Figures 19. (a) Complex compound $[Cu(II)(L_2)(H_2O)(NO_3)_2]$, (b) Polymeri -zation. [17]

Complex compounds copper(II) by ligand tridentat 1 imidazoles

Sarker et al., 2010 has been to synthesize a complex compound with Cu(II) as the central atom, 1-alkyl-2-(o-tioalkil) fenilazoimidazol as ligands tridentat, and SCN as bridging ligand. Molecular formula is [CuII (SetaaiNMe) $(SCN)_2$]. Imidazole donated three pairs of free electrons to bind together with the atom Cu(II). SCN as ligands bridge will connect between the molecules form a complex compound supermolecule.[18]



Figures 20. (Left) Chemistry Structure, (Right) Design of molecule structure [Cu^{II}(SetaaiNMe)$(SCN)_2$]. [18]



Figures 21. Left [Cu(biap)Br_2] and right [Cu(biap)$(NO_2)_2$]. [19]

Baretta et al., 2000 managed to synthesize two complex compounds with imidazole as a ligand tridentate namely, [Cu(biap)Br_2] and [Cu(biap)$(NO_2)_2$]. Complex compounds [Cu(biap)Br_2] has the shape of trigonal geometry bipiramidal with Br_2 in apical position and 3 nitrogen of ligands and anions bromide in equarorial. Hydrogen bonding occurs in bromine and imdazol-NH atom in the molecule itself. Complex compounds [Cu(biap)$(NO_2)_2$] asymetric shape. Can be seen in the image below ions Cu(II) in a position squer pyramidal with two imidazole and 1 amine, nitroten together with the oxygen on the nitrite on the state of equatorial and equal.[19]

SUMMARY

Imidazoles have high anti-HIV inhibitory activity, some derivates of imidazole reported drugs. Imidazole a ring substituted and pirimidine ring for potent inhibitory activity against RT. Copper is a bio-essential element and copper complexes have been extensively utilized in metal mediated DNA cleavage for the generation of activated oxygen species. Copper can potential for anti-HIV, because copper have inhibitor activity for HIV-proteinase. Copper can interact with donor atoms on a biological target via the formation of coordinate bonds rather than a combination of weaker intermolecular force such as H-bonding and chelator. The chelator has high stability complex to retain the copper ion in vivo and exchangeable ligands must be present to allow coordination of amino acid side chains.

ACKNOWLEDGMENTS

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Research Report

CELLULAR IMMUNITY ACTIVATION METHOD BY STIMULATING RD1 COMPLEX PROTEINS AS VIRULENCE MARKER ON *Mycobacterium tuberculum* TO ESTABLISH DIAGNOSIS ON TUBERCULOSIS AND LATENT TUBERCULOSIS INFECTION

Rebekah J. Setiabudi [1,a], Ni Made Mertaniasih [2], Didik Handijatno [3], Retno Asih Setyoningrum[4]
[1,2] Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.
[3] Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia.
[4] Department of Pediatrics, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.
Corresponding author : rebekahsetiabudi@gmail.com

ABSTRACT

This study was intended to invent a simpler and more affordable method to establish diagnosis on Tuberculosis (TB) and Latent Tuberculosis infection (LTBI). Similar to "Quantiferon TB Gold In Tube" (QFT-GIT) and T.SPOT.TB methods, the researchers also utilized "early secreted antigenic target 6kDa" (ESAT-6) and "cultur filtrate protein 10kDa" (CFP-10) proteins to be induced on the specimen. ESAT-6 and CFP-10 are commercial products used to induce interferon gamma (INF-γ) which were to be read using sophisticated and expensive equipment. This study was intended to conduct an analysis on effective cocktail protein modification, i.e. ESAT-6, CFP-10 and Ag85A/B/C, with high validity to detect cellular immunity activity through in vitro examination on peripheral blood monocyte cells of Tuberculosis-suspected patients or patients with latent tuberculosis infection. Peripheral Blood Monocyte Cells (PBMCs) activity on children tuberculosis patient or Latent Tuberculosis Infection (LTBI), adult tuberculosis patient or LTBI, which induced by cocktail protein modification and not induced, were analyzed microscopically. The activity of PBMCs on children and adult tuberculosis patient or LTBI induced by RD1 secretory proteins: ESAT-6, CFP-10, Ag85A/B/C was higher compared to PBMCs which had not been induced by the secretory proteins. Cellular debris and monocyte cells with abnormal shapes were found on PBMCs which had been induced by RD1 secretory proteins at 8th day after culture.

Key words : *cellular immunity activation, Region of Difference 1 (RD1) complex proteins stimulation, virulence markers, Mycobacterium tuberculosis, diagnosis on Latent Tuberculosis Infection (LTBI).*

ABSTRAK

Penelitian ini bertujuan menemukan inovasi metode yang lebih sederhana dan terjangkau dalam hal biaya. Hampir serupa dengan QFT-GIT dan T-SPOT.TB assay, penelitian ini juga akan menggunakan protein "early secreted antigenic target 6kDa" (ESAT-6) dan "cultur filtrate protein 10kDa" (CFP-10) untuk induksi pada spesimen. Kedua produk komersial tersebut, ESAT-6 dan CFP-10, telah digunakan secara komersial untuk menginduksi interferon gamma (INF-γ) yang kemudian akan dibaca dengan alat yang canggih dan berbiaya tinggi. Studi ini bertujuan melakukan analisis modifikasi cocktail protein yang efektif, yaitu ESAT-6, CFP-10 and Ag85A/B/C, dengan validitas tinggi untuk deteksi aktivitas imunitas seluler pada uji in vitro kultur sel monosit darah tepi dari pasien dengan suspek Tuberkulosis atau infeksi Tuberkulosis laten. Aktivitas sel monosit Peripheral Blood Monocyte Cells (PBMCs) pada pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi dengan modifikasi cocktail protein dan yang tidak diberi induksi, dianalisis secara mikroskopis . Aktivitas sel-sel monosit PBMCs dari pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C lebih tinggi dibandingkan dengan sel-sel monosit PBMCs dari pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang tidak diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C. Terdapat beberapa debris sel dan bentukan

abnormal dari sel monosit pada kultur hari kedelapan dari sel-sel monosit PBMC pasien tuberculosis aktif/tuberculosis laten pada anak/tuberculosis dewasa/LTBI yang diberi induksi campuran protein sekretorik RD-1 : ESAT-6, CFP-10, Ag85A/B/C.

Kata kunci: *aktivasi imunitas seluler, stimulasi protein kompleks RD-1, marka virulensi, Mycobacterium tuberculosis, diagnosis infeksi tuberculosis laten.*

INTRODUCTION

Tuberculosis (TB), an infectious contagious disease caused by *Mycobacterium tuberculosis* still becomes one of global health issues especially in developing countries. World Health Organization estimated that almost a third of world population had ever infected by TB with 8.7 million new cases found and annual mortality rate as much as 1.4 million.[1,2] According to Indonesian Ministry of Health, Indonesia positioned the fourth rank among countries with highest TB cases and pulmonal TB had infected many individuals at productive age and become the second highest cause of death in Indonesia.[3]

The burden of TB in Indonesia is increasing as the increase of new resistant TB cases found in Indonesia. The new TB cases are resistant to standard medication regiment, such as *Multi Drug Resistant TB/MDR-TB,* followed by *Emerging Extended Drug Resistant TB/XDR-TB,* and *Extreme Extended Drug Resistant TB/XXDR-TB*. The load of TB in Indonesia becomes greater as the emerge of co-morbid TB cases with Human Immunodeficiency Virus (*TB-HIV*).[1,3] Immunocompromised condition caused by HIV might improve the risk of TB infection, reactivation of dormant *Mycobacterium tuberculosis* on Latent Tuberculosis Infection (LTBI) patients, and mortality rate caused by TB.[1,4,5] WHO estimated about 10% of LTBI could develop to TB.[2,6] Therefore, tuberculosis was the main opportunistic infection in TB endemic areas, including Indonesia.[2,4]

Lower new TB cases found compared to expectancy rate of TB cases indicated that there were many TB cases which is happening on communities remained unidentified and has not been covered by TB governance.[7] One of the causes of this problem was inaccurate and improper implementation of TB diagnosis establishment in Indonesia. In order to establish diagnosis on TB, the patient should undergo laboratory examination in health care institutions. However, many people living in remote areas in Indonesia found difficulties in accessing health facilities due to distance and length of time to be taken to reach health care centers. This problem became harder because of bad roads, limited means of transportation, limited electricity availability and coverage, limited health care facility, and low quality and quantity of human resources owned by health care facilities in Indonesia.[3]

The first important step to be taken in conducting an effective and efficient TB prevention effort is improving case finding by applying proper method and establishing accurate diagnosis on TB. A quick and accurate TB diagnosis is the foundation in determining adequate medication.[2] Quick and accurate detection and identification on TB infection enables quick and adequate medication given to the patient in order to prevent pulmonal tissue damage and transmission of the disease. However, confirming diagnosis on tuberculosis was not easy, especially on primary tuberculosis cases, extra-pulmonal tuberculosis cases (i.e. *pleural tuberculosis, cerebrospinal fluid (CSF), pericardial tuberculosis,* and *ascetic tuberculosis*), TB on children, and TB-HIV co-infection cases. Confirming TB diagnosis becomes more difficult as the improvement of *Non-tuberculous Mycobacteria (NTMs)* infection prevalence.[8]

Common methods to diagnose TB such as through microscopic examinations on dyed *Acid Fast Bacilli (AFB)* and specimen culture require sophisticated health facility and skilled health facility operator. These methods also possess some disadvantages. Microscopic examination on dyed AFB has limited sensitivity and specificity while specimen culture takes too long to produce its results. The results of specimen culture can only be achieved 2-8 weeks later.[8] Tuberculin Skin Tests (TSTs) have been used worldeide for more than a century as an aid in diagnosing both LTBI and active tuberculosis but a valid TSTs requires proper administration by the Mantoux method with intradermal injection of 0,1ml of tuberculin-purified protein derivative (PPD) into the volar surface of the forearm. In addition, patients must return to a health-care provider for test reading, and inaccuracies or bias can exist in reading the test.[9] The most recent method, *Interferon Gamma Release Assays (IGRA)* is quite promising.[7] This method offers accurate sensitivity and specificity in shorter time period.[10,11] However, this method is still quite expensive and requiring sophisticated instruments making this method quite hard to be applied in health care centers in Indonesia. A quick, accurate, and affordable method to diagnose active tuberculosis infection and Latent Tuberculosis Infection (LTBI) becomes an urgent need.[12]

This study was conducted to invent a simpler and more affordable method in diagnosing tuberculosis infection and Latent Tuberculosis Infection (LTBI). Similar to QFT-GIT and T-SPOT.TB assay, in this study, the researchers use ESAT-6 and CFP-10 proteins to be induced into patient *Peripheral Blood Monocyte Cells (PBMCs)* specimen.[13,14] ESAT-6 and CFP-10 are commercial proteins which are used to induce interferon gamma (INF-γ) and the result will be examined using sophisticated and expensive equipment.[15,16] Different from previous studies, in this study the researchers do not only use ESAT-6 and CFP-10 proteins but also use

other proteins from *Region of Difference 1 (RD1)* protein family, such as Ag85A/B/C to be induced into suspected TB patient and LTBI patient's PBMCs suspension through in vitro manner.[17,18] Specific proteins from RD1 family can be found on all virulent strains and all clinical isolation of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.[19,20] RD1 protein family perform enzymatic function, namely to metabolize lipid on *Mycobacterium tuberculosis* cell wall.[21] These proteins were associated to virulence and immunogenicity of *Mycobacterium tuberculosis*.[22,23] After being induced, the monocyte cells undergo microscopic examination under light microscope to examine the activation of monocyte cells.

MATERIAL AND METHOD

This study was categorized as laboratory experimental research. This study was conducted by comparing treatment groups consisting of Peripheral Blood Monocyte Cell (PBMCs) culture sample collected from TB-suspected patients and LTBI patients which had been induced by RD1 secretory protein compounds and control group consisting of monocyte cell which had not received treatment (i.e. RD1 secretory protein induction). PBMCs suspension samples were collected from children TB patients/LTBI,[24,25,26] and adult TB patients/LTBI based on random consecutive method.[27,28] The samples were collected by collecting 5-10 mL median cubital vein blood based on vena puncture method using syringe. The collected blood was put into flasks containing heparin anti-coagulant. The flasks were shaken slowly to mix the blood with the anti-coagulation agent and preventing the blood from coagulation. PBMCs preparation was conducted based on Ficoll-Histopaque 1077 technique. PBMCs culture was incubated for 7 to 10 days enabling the monocyte cells to differentiate into macrophage. This process produced 10^5 macrophages in each well. At the 4th day, treatment on PBMCs samples was conducted namely by inducing ESAT-6, CFP-10, Ag85A/B/C protein compounds into several wells while the viability of PBMCs was observed using inverted microscope and Giemsa colorization. RD1 secretory protein compounds (ESAT-6, CFP-10, and Ag85A/B/C) were prepared by culturing *Mycobacterium tuberculosis H37Rv* on Lowenstein Jensen medium. The culture was incubated for 3-4 weeks enabling the culture to reach its logarithmic phase. 1 ose of *Mycobacterium* colony was collected and put into 10 mL Middle Brook 7H9 medium which had been prepared before. The suspension was incubated in CO_2 incubator at 37 °C for 2-3 weeks with loosened cap. After 2-3 weeks incubation period, the flasks containing *Mycobacterium tuberculosis* cultured in Middle Brook 7H9 medium were centrifuged for 30 seconds-1 minute until the mixture became homogenous. The mixture was rested for an hour until sedimentation formed. 200 µL supernatant was collected and used as "treatment" (TX) in this study.

RESULTS AND DISCUSSION

This study was conducted from November 2014 to February 2015. The samples of this study consisted of Peripheral Blood Monocyte Cells (PBMCs) samples collected from healthy patients with negative Tuberculin Skin Test (TST) result (K2), and PBMCs samples collected from child tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI (K1). Treatment (TX) was given to several K1 samples at the fifth day after incubation. The results were compared with K1 samples which had not received treatment. PBMC culture was cultivated daily by feeding (i.e. changing the medium daily). Microscopic examination was conducted at the 8th day.

Table 1. Results of Peripheral Blood Monocyte Cells Activity Examination

Result of Examination	K2	K1 (Without TX)	K1 (Receiving TX)
Amount of Monocyte/100lp	11.56	49.22	80.33

Note:
K1: PBMCs samples collected from children tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI.
K2: PBMCs samples collected from healthy individual with negative Tuberculosis infection.
TX: Treatment (ESAT-6, CFP-10, Ag85A/B/C secretory protein compounds induction).

Table 2. Results of Peripheral Blood Monocyte Cells Qualitative Analysis

Microscopic Examination	K2	K1 (Without TX)	K1 (Receiving TX)
Cellular Debris, Abnormality in Monocyte Cells Morphological Appearance	Negative	Negative	Positive

Note:
K1: PBMCs samples collected from children tuberculosis patients/LTBI, and adult tuberculosis patients/LTBI.
K2: PBMCs samples collected from healthy individual with negative Tuberculosis infection.
TX: Treatment (ESAT-6, CFP-10, Ag85A/B/C secretory protein compounds induction).

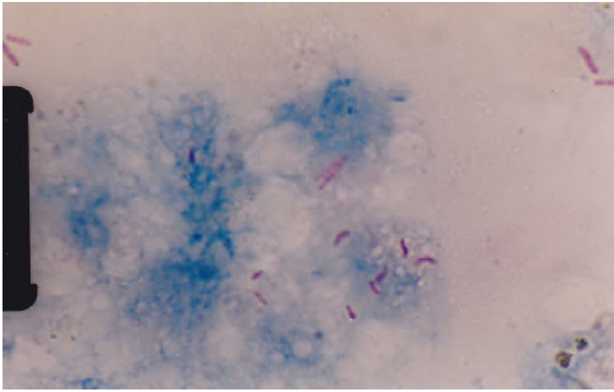

Figure 1. Result of microscopic analysis on *Mycobacterium tuberculosis H37Rv* suspension preparation cultured in Middle-Brook 7H9 medium (ZN, 100x). Acid Fast Bacilli are identified as red thin bacillus.

Mycobacterium tuberculosis H37Rv suspension cultured in Middle-Brook 7H9 medium, was stained by Ziehl Nielsen staining for detection. It is seen that the morphology of Mycobacterium tuberculosis is red thin rod/bacillus.[29]

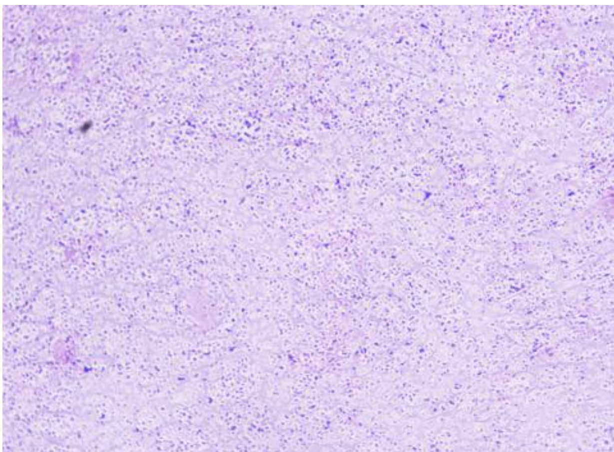

Figure 2. Patient PBMCs culture preparation at the 5th day (before receiving treatment) (Giemsa coloration, 10x): Monocyte cell started growing (indicated by dark blue nucleus with round structure).

PBMCs was cultured and incubated for 7 to 10 days enabling the monocyte cells to differentiate into macrophage. But we do the feeding of the PBMCs culture day by day to keep them alive.

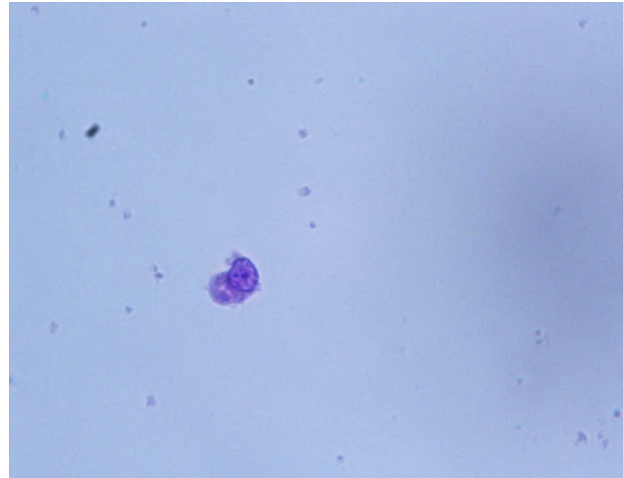

Figure 3. Patient PBMCs culture at the 5th day (Giemsa coloration, 100x) before treatment: Dark blue kidney-shaped monocyte nucleus started developing into macrophage.

Monocyte cell start to become a macrophage by developing the pseudopodia from its cellular membrane.

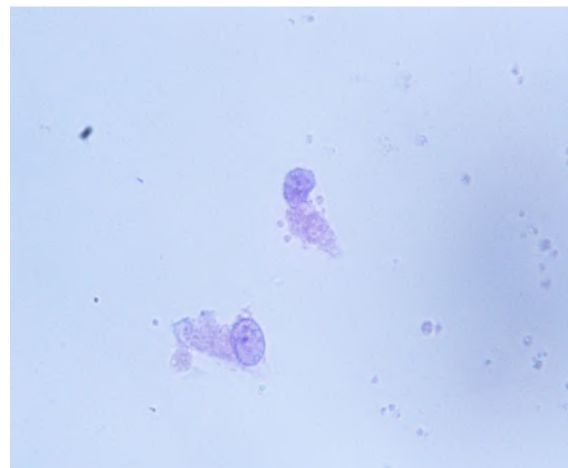

Figure 4. Patient PBMCs culture at 8th day (after treatment) (Giemsa, 100x): Monocytes are interacting with macrophage.

After treatment, there were a lot of active macrophages formed from monocytes. It is seen that the two macrophages is active and did the engulfment.

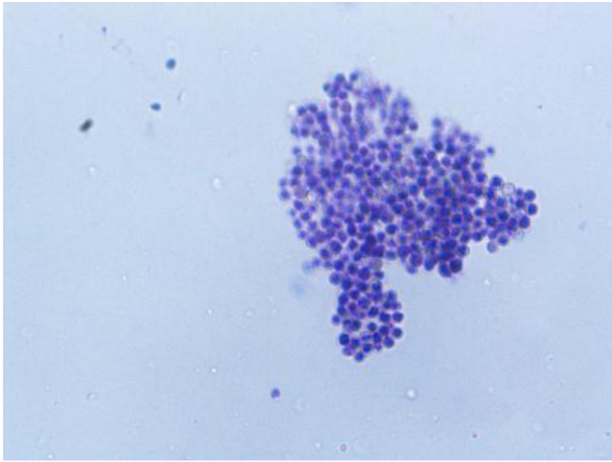

Figure 5. Patient PBMCs culture preparation at 8th day (after treatment) (Giemsa, 100x): Monocyte-lymphocyte cells are interacting with *M. tuberculosis* infected macrophage.

After treatment, there were many monocyte and lymphocyte cells came to interact with macrophages which had been infected by *Mycobacterium tuberculosis*.

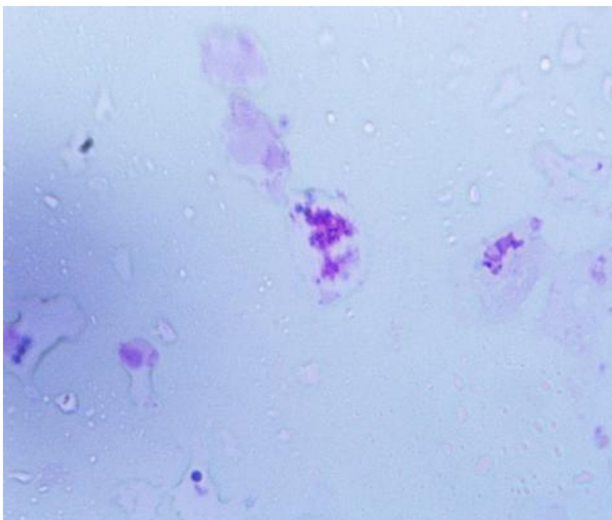

Figure 6. Patient macrophage preparation at 8th day (after treatment) (Giemsa, 100x): macrophage nuclei undergoing pyknosis (probably caused by autophagy).

After treatment, some of macrophages became pyknosis. Pyknosis is the irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis.

Interaction between macrophage and *Mycobacterium tuberculosis* (i.e. the role of macrophage as response made by host) was very important in Tuberculosis infection. Complement receptors (CR1, CR2, CR3, and CR4), mannose receptors (MR), and other molecular receptors on the surface of the cells played significant role in binding the microorganism to the phagocytes. Interaction between phagocyte molecular receptors and mycobacteria might be mediated by *Lipoarabinomannan (LAM)*, glycoprotein found on the surface of mycobacteria. *Prostaglandin E2 (PGE2)* and *Interleukin 4 (IL4)*, cytokines produced by *T-helper 2* (Th2) cells, expression regulation, the function of MR and CR receptors, and interferon γ (INF-γ) could reduce receptors expression resulting in reducing the ability of mycobacteria to attach to macrophage cells. Surfactant proteins, CD14 receptors, and scavenger receptors also functioned in mediating mycobacteria attachment.[30]

Microorganisms underwent phagocytosis were to be degraded through hydrolysis at acid condition after phagolysosome fusion. This process indicated a significant antimicrobial mechanism performed by phagocyte cells. Meena LS and Rajni (2010) proposed a hypothesis stating that phagolysosome fusion inhibition referred to a mechanism in which *Mycobacterium tuberculosis* survived in macrophage cell.[31] Previous studies reported that *mycobacterial sulphatides*–a derivative of *multiacylatedtrehalose 2-sulphate*- had an ability to inhibit phagolysosome fusion.[32,33] Previous in vitro studies showed that *Mycobacterium tuberculosis* produced a huge amount of ammonia which could be the factor affecting this inhibition process.[34,35] There were several functions of macrophage antimicrobial effectors including improving Reactive Oxygen Intermediates (ROI), Reactive Nitrogen Intermediates (RNI), and other mechanisms mediated by cytokines.[30] Hydrogen peroxide (H_2O_2), one of ROI produced by macrophage through oxidative reaction was identified as the first molecular effectors affecting mycobactericidal effect of mononuclear phagocyte cells. Previous laboratory researches showed that *Mycobacterium tuberculosis* infection might induce accumulation of macrophage on pulmonal tissue and H_2O_2 production. However, H_2O_2 production improvement by alveolar macrophage cells was not specific on TB infection. Moreover, alveolar macrophage cells produced less H_2O_2 compared to blood monocyte cells.[4]

Through interferon gamma (INF-γ) and Tumor Necrosis Factor alpha (TNF-α), phagocyte cells produced nitric oxide (NO) and other RNI through Inducible Nitric Oxide Synthase ($INOS_2$) using L-arginine as substrate. The significance of these toxic nitric substances as host immune response against *Mycobacterium tuberculosis* had been proven in in vitro examination, especially by using murine.[30]

Another mechanism as the result of interaction between macrophage cells and *Mycobacterium tuberculosis* was antimicrobial effect mediation by IFN-γ and TNF-α. Previous reports indicated human IFN-γ macrophage effect on *Mycobacterium tuberculosis* replication was varied from inhibition to enhancement. 1,25-$(OH)_2D_3$ itself (or combined with IFN-γ and TNF-α) might activate macrophage to inhibit and kill *Mycobacterium tuberculosis* inside human body.[36]

Other potential mechanism associated with macrophage defense response to *mycobacterium tuberculosis* was

apoptosis or "Programmed Cell Death. Lee et al (2009) showed that apoptosis by macrophage might reduce the viability of *Mycobacterium tuberculosis*.[37]

Until recently, biomolecular processes taking place in macrophage after *Mycobacterium tuberculosis* infecting the host andhow the bacteria survive on these processes are still being studied.[38] This study is attempted to compare the monocyte cells of healthy individuals and TB patient monocyte cells and TB patient monocyte cells which have been induced by ESAT-6, CFP-10, and Ag85A/B/C secretory protein compounds through microscopic examination. The results indicated improvement on monocyte cell activity of TB patient peripheral blood sample after secretory protein induction compared to the activity of TB patients which had not received secretory protein compounds. The result also showed that monocyte cell activity of TB patient was higher than healthy individual (control group). The result also indicated abnormal monocyte cells morphological appearance of TB patient monocyte cells after receiving ESAT-6, CFP-10, and Ag85A/B/C induction. The abnormal appearance was probably caused by macrophage/monocyte cells undergoing autophagy.

CONCLUSION

Monocyte cells activity of child tuberculosis patient, children with LTBI, and adult tuberculosis patient was higher than healthy individual monocyte cells activity.

Monocyte cells activity of child tuberculosis patient, children with LTBI, and adult tuberculosis patient receiving treatment (i.e. RD1 secretory proteins induction) was higher compared to monocyte cells which did not receive treatment.

Cellular debris and abnormal monocyte cell appearance were found at 8th day examination on child tuberculosis patient, children with LTBI, and adult tuberculosis patient monocyte cells sample after receiving RD1 secretory proteins (ESAT-6, CFP-10, and Ag85A/B/C) induction.

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Case Report

Mycobacterium leprae BACILLEMIA IN BOTH TWINS, BUT ONLY MANIFEST AS LEPROSY IN ONE SIBLING

Netty Sukmawati,[1] Indropo Agusni,[1] M. Y ulianto Listiawan,[1] Cita Rosita S Prakoeswa,[1] Dinar Adriaty,[2] Ratna Wahyuni,[2] Iswahyudi[2]
[1] Dept. Of Dermatology, School of Medicine, Universitas Airlangga, Surabaya, Indonesia.
[2] Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

ABSTRACT

Leprosy in twins is rarely reported. A 19 years-old male student, from Lamongan district, was diagnosed as Multibacillary (MB) leprosy in the Skin and STD Clinic of Dr. Soetomo General Hospital Surabaya. Multiple anesthetic skin lesions were found, but the bacteriologic examination was negative for Acid Fast Bacilli (AFB). Histopathology examination support the diagnosis of BL type of leprosy. His twin brother that has been lived together since born until present seems healthy without any complaints of skin lesions and have no signs of leprosy. When a serologic examination for leprosy was performed, a high anti PGL-1 antibody level was found in patient (IgM anti PGL-1 2937 and IgG anti PGL-1 3080 unit/ml) while his healthy twin brother showed only low level (IgM 745 and IgG 0 unit/ml). Interestingly when a PCR study was performed to detect M.leprae in the blood, both of them showed positive results. Using the TTC method, a genomic study of for M.leprae, it is revealed that both samples were identic (27x TTC repeats). According to patient's history, he had a traffic accident and got a wound in the knee seven years ago, while the skin lesions seems started from this area around three years ago before it spread to other parts of the body. The patient was treated with Multi-drug therapy (MDT) while his sibling got a prophylactic treatment for leprosy. After 6 months of treatment, the leprosy skin lesions were diminished and the serologic anti PGL-1 has been decreased. His healthy brother also showed a decrease in anti PGL-1 level and no skin signs of leprosy.

Key words: *leprosy, twin, bacillemia, PCR, prophylactic treatment*

ABSTRAK.

Penyakit kusta pada pasien bersudara kembar merupakan peristiwa yang jarang terjadi. Dilaporkan seorang pemuda berumur 19 berstatus mahasiswa yang datang berobat ke RSUD Dr Soetomo Surabaya dengan keluhan bercak di kulit kaki, badan dan muka. Pasien berasal dari daerah Lamongan dan bersaudara laki-laki kembar, tetapi dalam keadaan sehat. Diagnosa penyakit kusta ditegakkan berdasarkan lesi kulit yang anestesi, meskipun tidak ditemukan Basil Tahan Asam (BTA) dari lesi kulit. Pemeriksaan histopatologis menunjang diagnosa yang sesuai dengan kusta tipe BL. Saudara kembarnya yang telah tinggal bersama sejak kecil tidak menunjukkan adanya lesi kulit ataupun BTA. Pada pemeriksaan serologi anti Phenolic Glycolipid-1 (PGL-1) pada pasien didapatkan kadar yang tinggi (IgM 2937 u/ml dan IgG 3080 u/ml) sedangkan saudara kembarnya menunjukkan IgM anti PGL-1 745 u/ml, sedangkan IgGnya 0. Yang menarik adalah saat dilakukan pemeriksaan PCR untuk mendeteksi adanya M.leprae dalam darah, ternyata keduanya sama-sama menunjukkan hasil PCR yang positif. Selanjutnya dengan metode TTC dilakukan studi genomic dari M.leprae yang ditemukan. Hasil sekuensing pengulangan TTC menunjukkan bahwa ke 2 sampel tersebut identik (27x pengulangan TTC). Pasien diobati dengan obat Multi-drug Therapy (MDT) sedangkan untuk saudara kembarnya diberikan obat pencegahan kusta. Evaluasi setelah 6 bulan menunjukkan perbaikan klinis pada pasien dan penurunan titer antibodI anti PGL-1, sedangkan saudara kembarnya tetap tidak menunjukkan adanya gejala kusta serta semakin rendahnya titer antibodi.

Kata kunci: *kusta, saudara kembar, basilemia, PCR, terapi pencegahan*

BACKGROUND

Leprosy is a chronic disease caused by Mycobacterium leprae that primarily effects the peripheral nerves and secondary affects the skin and other organs.[1] Transmission leprosy dependent on immunological status and susceptibility, household contact, the environment and social conditions such as economic status, lacking of ventilation at home or poor hygiene.[2] Genetic factors are also to be a important factor in transmission of leprosy disease. Studies suggest that, among monozygotic (identical) twins if one had leprosy, the other almost always had leprosy, while this was not the case with dizygotic twins.[3] It is also influenced by human leukocyte antigen (HLA) that affect susceptibility.[4]

The main transmission route of M.leprae is droplet infection, but transmission such as skin contact, through the placenta during pregnancy, breast-feeding and trauma should not be ruled out even though there is no conclusive evidence.[5]

WHO recommends the Multi-drug Therapy (WHO-MDT) regiment for leprosy and the program have began since 1980 in Indonesia.[6] Although most of leprosy cases have been treated, there are still new leprosy cases were detected every year, indicating that transmission of leprosy still occurred in the community.[7] One of the reasons for explaining the continuing of new detected leprosy cases is the non-human resource of M.leprae. Since the human source (leprosy patients) are already treated by MDT and become non-infectious anymore, the role of non-human resources should be kept in mind.. These non-human resources including water, soil or other contaminated agents.[7] Several studies reports existence of viable M.leprae outside the human body. Detection of viable Mycobacterium leprae (RNA M.leprae) found in soil samples in Ghatampur India.[8] DNA M.leprae also found in water sources (wells) in along coast of East Java[9] M.lepra in soil and wells water were reported in leprosy endemic areas of East Java, including Lamongan Regency.[10]

CASES

Twins (Y and D) , 21 years-old students, unmaried, from Lamongan, visited the Skin and VD Clinic of Dr Soetomo General Hospital Surabaya. One sibling, Y, complained anasthetic red patch, which firstly appeared in front of right knee since 3 years ago. Y and D was born on 1994 in Payaman, Solokuro, part of Lamongan distric. Both of them were normaly born in one placenta (monozygotic). They spent time together in one house and sharing one bedroom since childhood. When they were 13 years old, they had junior school in Sendang agung village, Paciran, part of Lamongan district. In 2012 they became students in Malang and still lived in one the dorm rooms.

In 2007, Y was 14 years old, he got accident falling to the ground in Lamongan. He got trauma behind the right knee. At that time the wound just treated with antiseptic and healed. Six years later, in 2013, Y complained red patch which first appeared in front of the right knee. He went to a doctor and got some medications but the skin lesions still persist. Then the patient and his sibling visited the Out patient Clinic of Dr. Soetomo Hospital Surabaya.

Figure 1. Twins A. Y (leprosy patient) B. D (healthy twin).

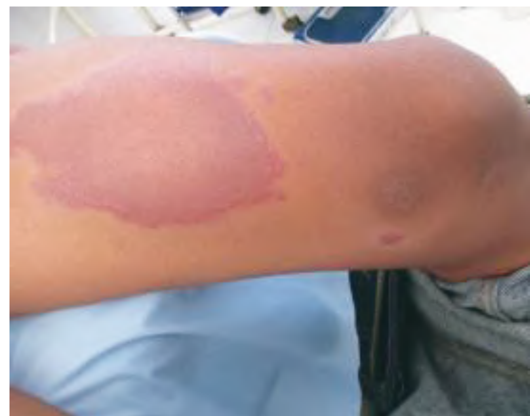


Figure 2. A. First anesthetic lesion on the knee

Multiple anaesthetic skin lesions were found over the right extremity and face. Negative result of skin slit smear for Acid Fast Bacilli (AFB) were noted from bacteriological examination using Ziehl Neelsen staining. Skin biopsy from the skin lesion at the right extremity revealed a BL type leprosy. Serological examination (ELISA anti PGL-1 antibody) serology to both of twins, ELISA results of IgM anti PGL-1 in Y patient was 2937 unit/ml and IgG anti PGL-1 was 3080 unit/ml. In the other health twin, serology results showed levels of IgM anti PGL-1 was 745 unit/ml and IgG anti PGL-1 was 0.

Skin biopsy from the skin lesion at the right extremity revealed a BL type leprosy. (Figure 3 & 4)

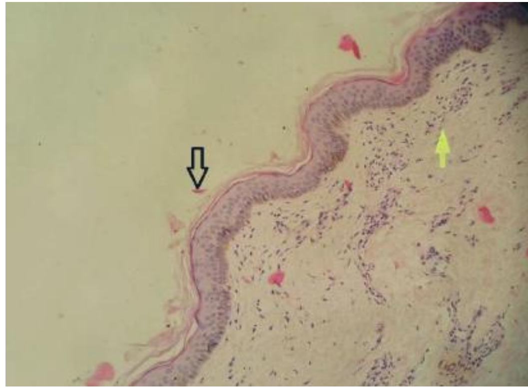


Figure 3. Epidermal atrophy, flattened rete ridges and grenz zone were observed (H/E 400x)

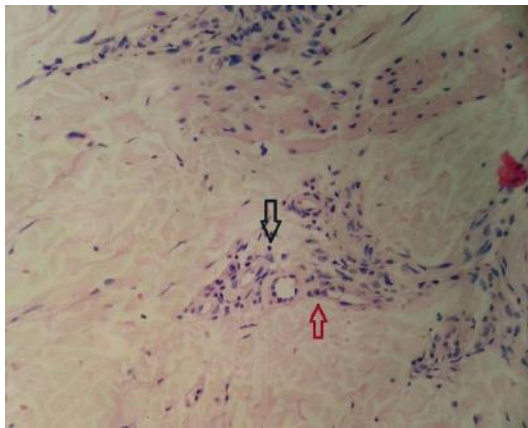
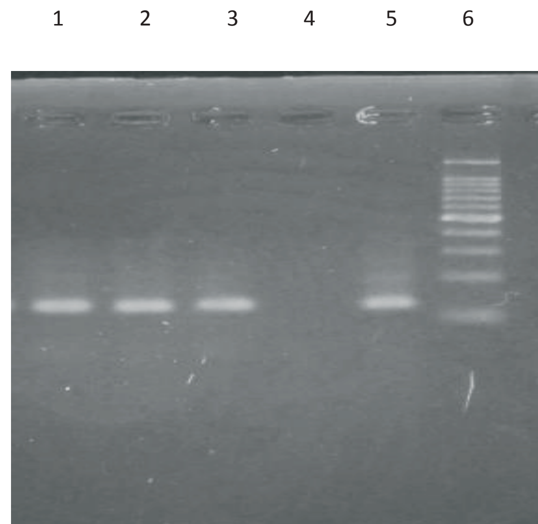


Figure 4. Dilated capillary blood with lymphocyte infiltration. . (H/E 400x)

Polymerase Chain Reaction (PCR) study was performed to the bloods of the twin, using the LpF and LpR nested primers to the bloods of the twin (Figure 5)



Note :

1. Pbmc from leprosy patient (Y)
2. Pbmc from healthy sibling (D)
3. Skin lesion of patient (Y)
4. Neg Control
5. Pos Control – M.leprae Thai53
6. 100bp DNA ladder

Figure 5. PCR results from blood and skin lesion (LpF –LpR nested primers) .

Further study was conducted to compare the genomic pattern between the two M.leprae DNA from the amplicon

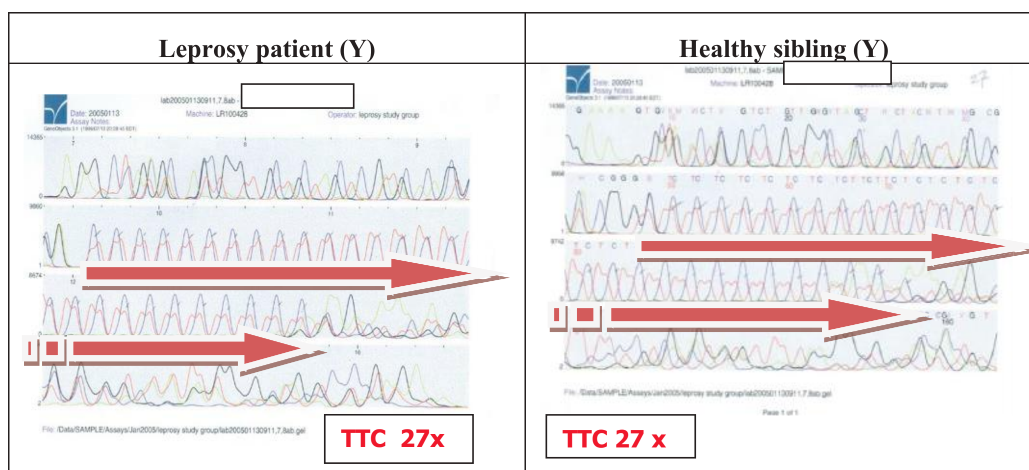


Figure 6. Direct sequencing of TTC area from both samples and number of TTC repeats

products of PCRresults . (Figure 6). Using the TTC method, the number of TTC repeats from both of samples were similar, 27x repeats, which indicates the two samples were identic or similar strain.

The examination results of these twins can be summarized as follows :

Data	Mr. Y (leprosy patient)	Mr. D (healthy siblings)
Skin lesions	Multiple anesthetic macules	No skin lesions
Bacterial examination (AFB)	Negative	Negative
Histopathology from skin lesion	BL type of leprosy	Not done
Serology (anti PGL-1)	IgM 2937 IgG 3080 u/ml	IgM 745 IgG 0 u/ml
PCR from skin lesion	Positive	Not done
PCR from blood (pbmc)	Positive	positive
Direct sequencing TTC area	27 x repeats	27 x repeats

The leprosy patient (Mr. Y) was treated with Rifampicine, Dapsone and Lamprene (WHO-MDT regiment) for 12 months while his healthy sibling was treated with a prophylactic dose of Rifampicine and Ofloxacine for two weeks. After six months later, the skin lesions disappear and the titer of anti PGL-1 antibodies were decreased. His healthy sibling does not develop any sign of leprosy and the anti PGL-1 titer became normal.

DISCUSSION

Leprosy in twin is relatively rare and seldom reported in the literature. Chakravartti & Vogel (1973) conducted an epidemiologic study leprosy in twins. Among 62 pairs of monozygotic twins and 40 pairs of dizygotic, they found that the monozygotic twins have a greater risk to get leprosy if the sibling affected the disease.3 Several studies reported several genes and substance may have a role in the susceptibility to leprosy, including HLA, TAP2, VDR, PTPN22 in adaptive immunity and NRAMP1, TLR2,MICA etc. in innate immunity.[4]

In our case, they are monozygotic twin which is theoretically will have a similar pattern. They live together since birth until adolescent in leprosy endemic area of Lamongan. This area has been known as leprosy endemic area in East Java since a long time ago.11 If the source of infection is the same, usually via droplet infection, they will got the same exposures and same long time duration. Then the incubation period will be the same and both of them will manifest leprosy on the same time. But in fact, leprosy manifest only in one sibling and the different life experience between them is the traffic accident seven years previously. The site of the first skin lesion of leprosy was very close with the scar of the wound during the accident three years ago. It might be possible that M.leprae entered the body via the wound and then spread to other organ. Non-human resource of M.leprae have been reported from some leprosy endemic areas and also some of them found the viable M.leprae from the soil and water.12. In our case, Mr. D who got traffic accident probably infected by the bacilli from environment, which become manifest leprosy after 4 years. The diagnosis of leprosy in this case is confirmed by the typical anesthetic skin lesions and histopathological examination. Although the other cardinal signs of leprosy (peripheral nerves enlargement and the present of Acid Fast Bacilli / AFB) was negative, the PCR results showed that the specific DNA of M.leprae was present in the skin lesion and peripheral blood. The serological test result of Mr.Y supported the diagnosis of manifest leprosy (high titer of IgM and IgG anti PGL-1) while the antibody titer of Mr.D showed a low sero-positive result (IgM anti PGl-1 745 u/ml with cut off 605 u/ml) that indicated a subclinical leprosy. One can assume that the process of leprosy in Mr.D is still in the initial stage, which will progress to manifest leprosy within certain years ahead.13 The use of the TTC technique, one procedure of Variable Number Tandem Repeat (VNTR) method for genetic study of M.leprae. This technique was chosen because it is relatively easy, simple and relatively low cost.14 The results showed 27x TTC repeats in both samples indicated similar pattern of the strain, which means they were originated from one similar source.

After got the disease, Mr. D became a source of infection for his twin brother. Positive PCR test from the blood indicates that the healthy brother was in subclinical stage of leprosy. This stage will develop toward the manifest leprosy after certain years, if no prophylactic treatment was given.15 Up to present time. there is still no guidance yet about chemoprophylactic treatment in Leprosy, therefore the use of Rifampicine and Ofloxacine for the subclinical leprosy in this case was based on the author's experience.[16]

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Research Report

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

Prihartini Widiyanti[a,b,1], **Bambang Prajogo**[c], **Ni Putu Ermi Hikmawanti**[c]
[a] Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia
[b] Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, East Java, Indonesia
[c] Department of Pharmacognosy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia
Corresponding author: drwidiyanti@yahoo.com

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 $_{(0.05)} = \pm 2.365$, 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]

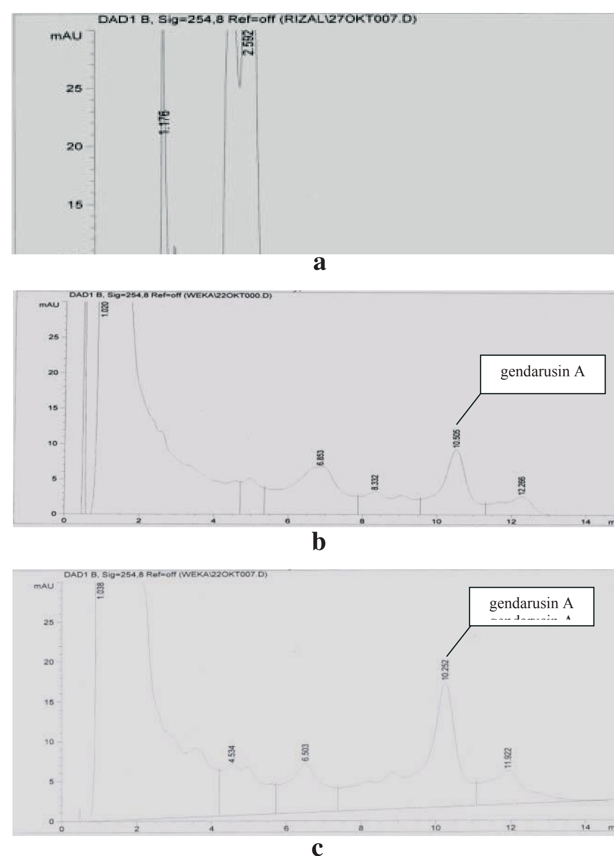

Figure 1. Chromatogram profile of (a) gendarusin A - 12.8 µg/ml solution; (b) gendarusin A in 5000.0 µg/ml of the fractionated-70% ethanol extract of *J. gendarussa* leaves; and (c) gendarusin A in 5000.0 µg/ml of the 70% ethanol extract of *J. gendarussa* leaves.

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.

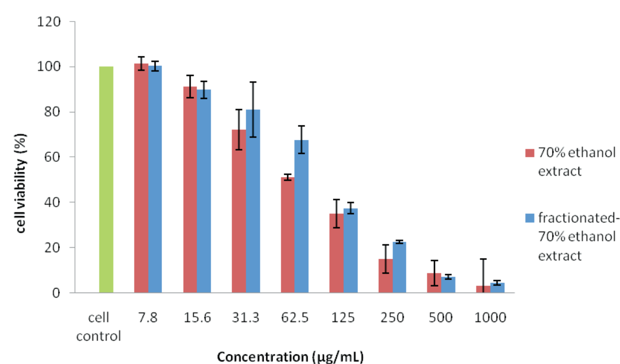

Figure 2. Comparison of the effect of various concentrations of fractionated-70% ethanol extract and 70% ethanol extract of *J. gendarussa* leaves on MOLT-4 cell viability with a 72 hr incubation period.

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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