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Anti-mycobacterial activity of methanol plants extract to against *Mycobacterium bovis* and *Mycobacterium smegmatis*

Aktivitas anti-mikobakterium ekstrak metanol tumbuhan untuk penghambatan *Mycobacterium bovis* dan *Mycobacterium smegmatis*

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Abstrak. *Syahputra G, Sari M, Kusharyoto W. 2016. Aktivitas anti-mikobakterium ekstrak metanol tumbuhan untuk penghambatan* Mycobacterium bovis *dan* Mycobacterium smegmatis*. Pros Sem Nas Masy Biodiv Indon 2: 182-187.* Tumbuhan merupakan sumber penting dalam pengobatan tuberculosis (TB). Bahan alam asli Indonesia yang berasal dari tumbuhan obat merupakan aset penting dalam pengobatan TB. Tujuan dari penelitian ini adalah untuk mencari senyawa anti-mikobakterium yang berasal dari ekstrak tumbuhan, seperti *Azadirachta indica*, *Zingiber americans*, *Desmodium triquetum*, *Clerodendron serratum*, *Caesalpinia sappan*, dan *Morus alba* dalam menghambat *Mycobacterium bovis* dan *Mycobacterium smegmatis* dengan menggunakan metode difusi cakram. Bakteri *M. smegmatis* merupakan bakteri yang paling sensitif dalam pengujian terhadap ekstrak tumbuhan. Ekstrak *D. triquertum*, *C. serratum*, dan *C. sappan* memperlihatkan adanya aktivitas anti-mikobakterium pada bakteri uji. Variasi konsentrasi ekstrak menunjukkan bahwa ekstrak metanol dari *D. triquertum* paling potensial sebagai anti-mikobakterium. Berdasarkan uji fitokimia, senyawa pada *D. triquertum* mengandung flavonoid dan tanin. Perlu dilakukan pengujian lanjutan untuk mengisolasi senyawa aktifnya sebagai anti-mikobakterium.

Kata kunci: Aktivitas anti-mikobakterium, ekstrak tumbuhan, difusi cakram, uji fitokimia, kromatografi lapis tipis, KLT

Abstract. *Syahputra G, Sari M, Kusharyoto W. 2016. Anti-mycobacterial activity of methanol plants extract to against* Mycobacterium bovis *and* Mycobacterium smegmatis*. Pros Sem Nas Masy Biodiv Indon 2: 182-187.* The plant is an important source of new drug for the treatment of tuberculosis (TB). Indonesian natural products derived from medicinal plants are important sources of TB therapeutics. This study aimed to screen for anti-mycobacterial activity derived from plants extract, such as *Azadirachta indica*, *Zingiber americans*, *Desmodium triquetum*, *Clerodendron serratum*, *Caesalpinia sappan*, and *Morus alba* in against *Mycobacterium bovis* and *Mycobacterium smegmatis* by using a disc diffusion method. *Mycobacterium smegmatis* is the most sensitive bacteria in treatment to plants extract. The extract of *D. triquetrum*, *C. serratum* and *C. sappan* showed the anti-mycobacterial activity in tested bacteria. Variation of extract concentration showed that the methanol extract of *D. triquetrum* is the most potential as an anti-mycobacterial. Based on a phytochemical assay, the compounds in *D. triquertum* contained flavonoids and tannins. It was needed to isolate the active compounds as an anti-mycobacterium.

Keywords: Anti-mycobacterial activity, disc diffusion, phytochemichal assay, plant extract, thin layer chromatography, TLC

INTRODUCTION

Tuberculosis (TB) is one of leading causes of morbidity and mortality globally. The global mortality rate stands at two million deaths per year with one third of the world population were infected by bacilli (CDC 2005; WHO 2007). The emergence of drug resistant strains of *Mycobacterium* is one of the major reasons contributing to the rise in global incidence of tuberculosis since 1980 (Lawn and Wikison 2006).

The treatment is available for TB has more than 20 types of drugs, and there are four types of first-line drugs, isoniazid, rifampicin, pyrazinamide, and ethambutol. Continued treatment such as using kanamycin, amikacin, capreomycin, and viomycin often used to maximize the treatment. Moreover there are kind of medicine seconde line bacteriostatic such as p-aminosalicyclic acid, ethionamide, and cycloserine but still have side effects. (Janin 2007). Isoniazid and ethionamide is mycolic acid synthesis inhibitors (Lei et al. 2000 and Banarjee et al. 1994), while cycloserine and ethambutol inhibiting the synthesis of peptidoglycan (Feng and Barletta 2003) and the cell wall arabinogalactan (Deng et al. 2003 and Belanger et al. 1996). Rifampicin and amikacin suppress pharmacological action by inhibiting synthesis of bacterial protein and RNA (Telenti et al. 1993, Busscher et al. 2005, and Maus et al. 2005).

Natural products have continued to provide new and important leads in the drug discovery process (Balunas and Kinghorn 2005). Natural products or their semi-synthetic

derivates have indeed provided a novel drug lead for tuberculosis therapy (Shu 1998). The plant kingdom can be looked at as an important source of new drugs for the treatment of TB because has an enormous chemical diversity (Gautam et al. 2007).

The study is confirm anti-mycobacterial from 6 Indonesian medicinal plants by disc diffusion method with variation of extract concentrations. Six potential medicinal plants are tested by the disc diffusion method were the result of screening 33 anti-mycobacterial candidate plant extracts. Screening is done by resazurin reduction assay perform with *Mycobacterium bovis* and *Mycobacterium smegmatis*. In addition to analyze the content of each potential medicinal plants conducted by phytochemical assay and the profile chemical compounds analysis by Thin Layer Chromatography (TLC).

Some of Indonesian plants have a potency for anti-bacterial activity, such as *Azadirachta indica*, *Zingiber americans*, *Desmodium triquetum*, *Clerodendron serratum*, *Caesalpinia sappan* and *Morus alba.* This research aimed to screen an anti-mycobacterial activity from six plant methanol extracts by a disc diffusion assay and to determine a profile of crude extracts by phytochemical assay and TLC. The drug discovery research is important for the treatment of TB, the drug design conduct with screening of chemical structure and treatment target by in silico method continued with in vitro and in vivo analysis.

MATERIALS AND METHODS

Plant samples

Leaves of *A. indica* (Azadirchta), *D. triquetum* (Fabaceae) and *C. serratum* (Verbenaceae), bark of *C. sappan* (Caesalpiniaceae), rhizome of *Z. american* (Zingiberaceae) and whole plant of *M. alba* (Moraceae) were collected from different regions in Indonesia between February and April 2015. Plant samples used in this study were presented in Table 1.

Extract preparation

The plant samples were dried and powdered by using a blender at room temperature before taken placed into a flask of Soxhlet apparatus for an extraction by using methanol. The crude solution was concentrated by a rotary evaporator. Furthermore, the plant extracts were storage in DMSO 2% up to 0.03 g/ml yield of extract at 4^{o}C (Taneja and Tyagi 2007).

Mycobacterial culture preparation

Mycobacterium bovis strain BCG and *M. smegmatis* were grown at a logarithmic phase (OD 595 0.5) in Middlebrook 7H9 broth supplemented with 10% OADC. Cultures were incubated (37^{o}C, 5% CO_2) in a humid environment with shaking for 1-2 days before diluted to a turbidity equivalent 5x10^4 cells (Taneja and Tyagi 2007).

Antibacterial activity

The antibacterial activity was screened by using a disc diffusion assay. The concentration was varied (100%, 75%, 50% and 25% in DMSO 2%) in each disc. For bottom layer, 6 ml medium Middlebrook 7H9 with supplement was taken place into petri dishes (14 cm diameter) until had a solid texture. As a top layer, 200 µl each of microbial test (*M. bovis* and *M. smegmatis*) were added into 6 ml medium of Middlebrook 7H9 with supplement and homogenized with slowly shaking. The medium with microbial test was taken place onto a bottom layer at petri dishes. Thirty microliters (30 µl) of variation of plant extract concentrations were applied on each paper disc (Whatman Grade AA discs, 6 mm diameter). The discs were air-dried and taken place onto the seeded top layer of agar plates. As a positive control, it was used 6 µl Rifampicin, while as a negative control it was used 6 µl DMSO 2%. The plates were incubated at room temperature for 24 hours. Antibacterial activity was expressed as the zone of inhibition (mm) produced by the plant extract compared with all of controls (Hussain et al. 2011).

Qualitative for phytochemical testing

The plant extracts were tested for qualitative phytochemical data for terpenoids, tannins, flavonoids, alkaloids, steroids, quinones and saponins (Edeoga et al. 2005; Chandrakala et al. 2012; Ram and Sinha 2015).

Terpenoids were tested by adding chloroform (1 ml) to the extract (1 ml) and then an equal volume of concentrated sulphuric acid was added. Formation of a bluish red coloration indicated the presence of terpenoids.

Tannins were tested by boiling the dried powdered extract (0.5 g) in water (20 ml) in a test tube and then 2 ml of 0.1 M $FeCl_3$ was added. Formation of a blue black coloration indicated the presence of tannins.

Flavonoids were tested by adding an ammonium solution (5 ml) into 1 ml of aqueous filtrate of extract followed by addition of sulphuric acid (2 ml). A yellow coloration indicated the presence of flavonoids.

Alkaloids were tested by mixing 50 g of simplicias powder with 250 ml of 1% sulphuric acid. It was allowed to stand and then filtered. Ten milliliters of filtrate was shaken and added into Meyer's reagent. The formation of a white precipitate indicated the presence of alkaloids.

Steroids (Salkowski test) were tested by mixing 2 ml of chloroform extract, 1 ml of concentrated H_2SO_4 acid was added carefully along the sides of test tubes. A red color produced in the chloroform layer confirmed the presence of steroids.

Quinones were tested by mixing 0.5 g of plant extract and then 1 ml of concentrated H_2SO_4 was added. The formation of red colour showed the presence of quinones.

Saponins were tested by adding a drop of sodium bicarbonate into a test tube containing 0.5 ml of aqueous extract. The mixture was shaken vigorously and kept for 30 minutes. A honey comb like froth was formed and it showed the presence of saponins.

Thin layer chromatography (TLC)

Six plant extracts were checked for chemicals profile by using a thin layer chromatography (TLC) on a silica gel plate G-60. As much as 0.05 g each of plant extracts was diluted in methanol. Every 2-5 µl of extracts was put on the

silica gel powder. The solvent system in TLC was performed with water: methanol: n-heksan: EtoAc (2:1.5:1:2, v/v/v/v). The spot was checked in 254 nm of UV lamp.

RESULTS AND DISCUSSION

Plant extractions

In this study, six plants extracts, namely leaves of *A. indica* (Azadirchta), *D. triquetum* (Fabaceae) and *C. serratum* (Verbenaceae), bark of *C. sappan* (Caesalpiniaceae), rhizome of *Z. american* (Zingiberaceae) and whole plant of *M. alba* (Moraceae) were screened for their anti-mycobacterial activity against *M. bovis* strain BCG and *M. smegmatis* by using a disc diffusion assay at room temperature (Table 1). The results of six plant extracts showed that the highest yield (27.44%) by extraction process was obtained from leaves of *D. triquetum* with liquid texture and dark green for the physical characteristics. The extract of *M. alba* was the lowest yield (2.3008%) with liquid texture and dark green color of extract.

The results of study showed that extracts from six plants against *M. smegmatis* activity. However, three from six plants extracts also against *M. bovis* activity. The activity was found in all of variation concentration of *D. triquetum* extracts, 100% *C. serratum* extract, and found in 100% and 75% *C. serratum* extracts. All of the extracts were active to against the Rifampicin resistant strain of *M. smegmatis* and *M. bovis*. Rifampicin is an indicator for multi-drug resistant mycobacterium, so the extracts might also to be active on MDR TB.

The plant of *D. triquetum* was reported that its extract ascertainable as an antiinflamatory, hepatoprotective and had an antioxidant activity (Kalyani et al. 2011a; Kalyani et al. 2011b). *Caesalpinia sappan* is one of the plants which have an anti-mycobacterial activity. In the other report, *C. sappan* has an antibacterial activity to against some of human pathogenic bacteria such as *Eschericia coli*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Bacillus cereus* and *Enterococcis faecalis* (Saravankumar and Helan 2013).

In this study, *C. sappan* had an activity for anti-mycobacterial until 25% plant extract to against *M. smegmatis* and 50% plant extract to against *M. bovis*. The other plant, *C. serratum* was one of potential plant to be an anti-mycobacterial source. The report was showed in Table 2. It had an activity to against a kind of mycobacterium test. In some of other reports, *C. serratum* had an antibacterial activity which potential to against gastrointestinal bacterial pathogens, such as *Salmonella paratyphi*, *S. enterica typhimurium*, *S. enteric*, *Streptococcus mitis*, *S. salivarous*, *Pseudomonas aeruginosa*, *Micrococci*, *Klensiella pneumonia* and *Bacillus subtilis* (Shukla et al. 2014). The pharmacological activity of *C. serratum* was reported as antioxidant, anticancer, antibacterial and anti-inflamatory (Singh et al. 2012). From the data (Table 2), *M. alba* was reported capable to against *M. smegmatis* and from the other report, its plant extract had an antibacterial activity to against *Staphyploccus aureus*, *Bacills subtilis* and *Escherichia coli* (Singh et al. 2013). The extract of *A. indica* was potential to against *M. smegmatis* and from the other report, it had an antibacterial activity to against *E. coli* and *S. aureus* (Mishra et al. 2013).

Antibacterial activity

To confirm the formation of antibacterial activity from the methanol plant extracts, the plant extract of each isolate was determined to against *M. smegmatis* and *M. bovis* as a microbial test (Table 2). The results were shown in Table 2 and confirmed the ability of all plant extracts to produce an antibacterial activity. The variation of concentrations of all plant extracts showed to be able to against *M. segmatis*. In the other side, some of variation of concentrations from plant extracts to be able to against *M. bovis*. Compared with an anti-mycobacterial activity of Rifampicin as a positive control, almost of plant extracts had a higher potential than Rifampicin. The assay aimed to find out the potential plant extracts to against *M. smegmatis* and *M. bovis* as a microbial test. The assay used four variations of concentrations from each plant extract (100%, 75%, 50% and 25%). Six plants extracts against *M. smegmatis* in 25% extracts concentration. However, only three plants extracts against *M. bovis*, they were *C. serretum, C. sappan* and *D. triquertum* (Table 2). All of variation concentrations of *Z. americans*, *A. indica* and *M. alba* extracts, there was no antibacterial activity for a microbial test. *Desmodium triquetum* had a potential plant extract to against *M. smegmatis* and *M. bovis* representative in diameter of inhibition zone in agar plate. The extract of *D. triquetum* had an antibacterial activity for all variations of extract concentration.

The plant extracts had a potential activity for anti-mycobacterial. It was showed from a zone of inhibitor of plant extracts compared than Rifampicin as an antibiotic to against *Mycobacterium*. Rifampicin was used in this study for a positive control for anti-mycobacterial drug. The target of Rifampicin in mycobacterium is β-subunit of RNA polymerase, in which it binds and inhibits the elongation of messenger RNA (Blanchard 1996). Some of potential plant extracts, such as *D. triquetum*, *C. sappan* and *C. serratum* suggested an investigation deeply in the future. An anti-mycobacterial activity from the plants has been known from other methods, such as a resazurin reduction assay. The principal of this method is a change of colour from resazurin to be resorufin to find out the activity of cell (O'Neill et al. 2014).

Phytochemical assay

Phytochemical assay aimed to find out some compounds of plant extracts, such as alkaloids, steroids, flavones, tannins, saponins, triterpenoids and quinones. The plant extracts were tested to a phytochemical qualitative assay for finding out the compounds of them. Table 3 showed that none of all the plants had nothing alkaloids and saponins. Almost all the plants had flavones, except *A. indica.* Quinones were found in only *C. sappan* extract. Triterpenoids were found in two from six plant extracts, i.e. *C. sappan* and *Z. american.*

Table 1. The yield of plant extractions

Botanical name			Weight		Yield (%)	Texture	Color
Family	Species	Local	Raw material (g)	Extract (g)			
Verbenaceae	*C. serratum*	Senggugu	50	6.5826	13.1652	Liquid	Dark green
Caesalpiniaceae	*C. sappan*	Secang	25	3.6743	14.6972	Sticky	Dark red
Zingiberaceae	*Z. americans*	Lempuyang	44	3.3492	7.6118	Solid	Brown
Fabaceae	*D. triquetum*	Kicongcorang	6.35	1.7427	27.4400	Liquid	Dark green
Azadirachta	*A. indica*	Mimba	50	5.8037	11.6074	Sticky	Dark green
Moraceae	*M. alba*	Murbei	50	1.1504	2.3008	Liquid	Dark green

Table 2. The inhibition zone of the plant extracts by using a disc diffusion assay to against the test mycobacterial.

Botanical name	Variation of concentration of plant extracts	The inhibition zone (mm)	
		M. smegmatis	*M. bovis*
Clerodendron serratum	100%	7	9
	75%	6.25	NA
	50%	5.25	NA
	25%	5	NA
Caesalpinia sappan	100%	13.25	8.5
	75%	11	6.5
	50%	9.5	NA
	25%	6.5	NA
Zingiber americans	100%	6.5	NA
	75%	6	NA
	50%	5.5	NA
	25%	5	NA
Desmodium triquetum	100%	12.75	14.75
	75%	10.25	13
	50%	9	10.5
	25%	5.5	9
Azadirachta indica	100%	6	NA
	75%	4.5	NA
	50%	4	NA
	25%	4.25	NA
Morus alba	100%	4.5	NA
	75%	5.75	NA
	50%	5.75	NA
	25%	4.75	NA
Rifampicin	2.10^{-5} g/ml	9,25	9
DMSO	2%	NA	NA

Note: NA = Non-activity

Table 3. Phytochemical compounds of plants

Botanical name	Phytochemical compounds						
	Alkaloids	Steroids	Flavones	Tannins	Saponins	Triterpenoids	Quinones
C. serratum	-	+	+	-	-	-	-
C. sappan	-	-	+	+	-	+	+
Z. americans	-	-	+	+	-	+	-
D. triquetum	-	-	+	+	-	-	-
A. indica	-	+	-	+	-	-	-
M. alba	-	+	+	-	-	-	-

Note: + = present, - = absent

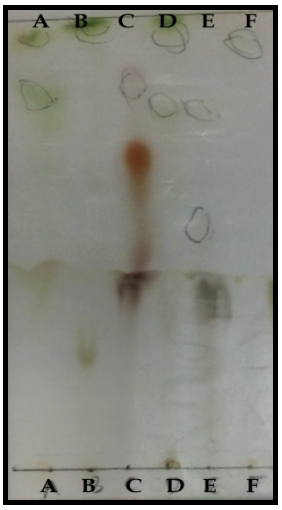

Figure 1. The result of TLC from each plant extracts. A = *A. indica*, B = *C. serratum*, C = *C. sappan*, D = *M. alba*, E = *D. triquetum* and F = *Z. amaricans*

The phytochemical assay for all of plant extracts (Table 3) showed some plants had the different component compounds. The compounds from a phytochemical assay might be used for first data to detect the pure compounds in plant extracts. Phytochemical assay of *C. sappan* contained flavonoids, tannins, triterpenoids and quinones. The report from Kumar and Nishteswar (2013) explained that the methanol extract of *C. sappan* contains flavonoids, phenolics, tannins, amino acids, proteins, carbohydrate, organic acids and saponins. Phytochemical assay for *C. serratum* resulted in steroids and flavonoids (Table 3).

The study reported the compounds in *C. serreatum* contained carbohydrate, flavonoids, phenolics, terpenes and steroids (Singh et al. 2012). For *D. triquetum*, the results (Table 3) of phytochemical assay were tannins and flavones. Reported by Thandar and Tun (2015), *D. triquetum* contained alkaloids, glycoside, flavonoids, saponins, amino acids and tannins. Table 3 showed that *M. alba* contained steroids and flavonoids. Reported by Singh et al. (2013), *M. alba* contained tannins and alkaloids. The other report from Salem et al. (2013) reported flavonoids, phenolics, saponins and tannins. For the phytochemical assay of *A. indica* showed that it contained steroids and tannins. The data (Table 3) showed that the extracts of *Z.*

americans contained flavonoids, tannins and triterpenoids. The component compounds of all plant extracts influence a pharmacological activity.

Thin Layer Chromatography (TLC)

Six plant extracts from extraction by Soxhlet with methanol solvent were tested by TLC in silica gel plate. Separation of the component of compounds in crude plant extracts by the solvent system was performed with water: methanol: n-hexane: EtoAc (2:1.5:1:2, v/v/v/v). The results of TLC showed some spots in silica gel plate. The difference of colour and amount of spots in the plate, representative a specific compound in each crude plant extracts. The extracts in Figure 1 were first data to find out the compounds in plant extracts. In the future, it should be an identification of the spots to determine the identity of the compounds. However, the pattern of spots from chemical was influenced by solvent, variation of concentrations and the working system of TLC.

The result of Thin Layer Chromatography (TLC) was as a preliminary test (Figure 1) from each plant extracts to find out the component compounds. The data should be continued with a chromatography or other chemical separation methods to get the pure compounds. From the data, it could be obtained to describe that *A. indica*, *C. serratum*, *C. sappam*, *Z. americans*, *M. alba* and *D. triquetum* contained some compounds based on the spots in the silica gel plate.

Six plant extracts had been studied to determine potential extracts to against *M. bovis* and *M. smegmatis*. Based on the anti-mycobacterial assay by a disc diffusion method, the potential plant extracts were *D. triquetum*, *C. serratum* and *C. sappan*. Three extracts were ascertainable to against both of microbial test. Based on the phytochemical assay, the plant extracts contained some compounds. The TLC results described that in the plant extracts contained many compounds for further treatment to get some pure compounds.

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