Antibacterial Activities of Extract n-Butanol-Methanol (1:1) Filtrat of the Fermentation Results of Streptomyces sp. B10 Against Mycobacterium tuberculosis H37Rv: An Experimental Study

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Antibacterial Activities of Extract n-Butanol-Methanol (1:1) Filtrat of the Fermentation Results of *Streptomyces sp.* B10 Against *Mycobacterium tuberculosis* H37Rv: An Experimental Study

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ABSTRACT

Background: *Streptomycessp.* is one of the sources of first choice antibiotic for tuberculosis treatment. *n*-butanol is the best solvent to extract antibacterial compound from fermented *Streptomycessp.* isolates.

Aim: To analyze anti-tuberculosis activity of extract n-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp*. B10 against *Mycobacterium tuberculosis* H37Rv.

Method: This was a laboratory experimental study. 3 comparisons of the type of solution were observed once a week for 3 weeks. Antibacterial activity test was analyzed from morphological observations on Ziehl-Neelsen staining, then minimum inhibitory concentration and minimum bactericidal concentration were determined.

Result: Bacterial growth began in the second week starting from a concentration of 10,50 ppm to a concentration of 1,31 ppm but a concentration of 43.000 ppm to a concentration of 5.375 ppm didn't show any bacterial growth. The results of Ziehl-Neelsen staining show a red and rod-shaped *Mycobacterium tuberculosis* H37Rv colonies at a concentration of 2.687,5 ppm to a concentration of 1,31 ppm and at a positive control. A concentration of 2.687,5 ppm was determined as minimum inhibitory concentration and a concentration of 5.375 ppm was determined as minimum bactericidal concentration.

Conclusion: Extract n-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp.* B10 at a concentration of 2.687,5 ppm to 5.375 ppm has antibacterial activity against *Mycobacterium tuberculosis* H37Rv. This extract can be used as an antibiotic formula for tuberculosis.

Keywords: *Streptomyces sp, n-butanol, tuberculosis, antibacterial activities*

Introduction

After first-line anti-tuberculosis drugs were released, the number of deaths was greatly reduced[1]. The characteristic of tuberculosis that rapidly resistant to drugs, and the ability of bacteria to survive is a major problem for public health[2]. In Indonesia, the total cases that reported in 2016 were 360.565 cases. In 2014, the prevalence of 660 per 100,000 population with a case incidence of 403 per 100,000 population[3]. The characteris of *Mycobacterium tuberculosis* is the ability to make a persistent infection that requires long-rm antibiotic therapy to cure tuberculosis patient[4]. Tuberculosis (TB) has become a curable disease due to the antibiotic invention[5-6].

One genus of *Actinomycetes* which contributes most of its active metabolites to antibiotics is *Streptomyces*, this genus occupies the first position on several antibiotics[7]. Antibiotic as anti-TB were discovered in 1943,known as Streptomycin[8].

Various strategies including bioinformatics are currently being tested to identify and improve

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vaccines against TB[9]. The researchers are undertaking and discovering new microorganisms which produce secondary metabolites[10-11]. Domestic production of drug ingredients has not been going well, and still depends on imports ingredients[12]. Five out of 15 isolates have been shown to have antibacterial activity from isolation using agricultural soil in Indonesia that planted with kale, spinach, and corn. One of the isolates known as *Streptomyces sp.* B10 which identified as *Streptomyces violaceusniger*[13,14]. Water fraction and *n*-butanol fraction from fermented *Streptomyces sp.* B10 has antibacterial activities but the minimum inhibitory concentration remains unknown[13]. Extract filtrate of the fermentation results of *Streptomyces sp.* B10 with *n*-butanol as solvent using KLT method with eluent methanol: water produces one stain[14]. Extract n-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp.* B10 showing antibacterial activity against *Escherichia coli*. *n-butanol* is a solvent which has been shown able to selectively extract antibiotic compounds from *Streptomyces* isolates with a large zone of inhibition[15] Methanol is a polar solvent that is mostly used because of its efficient penetration into the cell wall, so it produces more endocellular secondary metabolites[16].

This study aimed to analyze the activity of extract n-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp*. B10 against *Mycobacterium tuberculosis* H37Rv.

Method

This study was an experimental study conducted in the Microbiology Laboratory Faculty of Pharmacy Universitas Airlangga and Tuberculosis Laboratory Institute of Tropical Disease Universitas Airlangga in 2017. Bacterial samples were *Mycobacterium tuberculosis* H37Rv and obtained from Tuberculosis laboratory Institute of Tropical Disease Airlangga University Surabaya. *Streptomyces sp.* B10 isolates were obtained from the Microbiology laboratory collection, Pharmaceutical Chemistry Department, Faculty of Pharmacy Universitas Airlangga. This study was conducted by researchers and expert lecturers

The solution consists of 3 types: extract *n*-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp*. B10, 1,0 ppm rifampicin (RIF) solution and DMSO solvent, and positive control (Middlebrook7H10 medium and *Mycobacterium tuberculosis* H37Rv). Incubation temperature, pH, and incubation duration were controlled in this study. Laboratory testing procedures were carried out by using *Streptomyces sp.* B10 culture, fermentate the *Streptomyces sp.* B10, freeze dry the culture, extract the crude dry powder using *n*-butanol-methanol (1:1), evaporate the extraction, extract n-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp.* B10, and dilute. Antibacterial activity test was analyzed from morphological observations (shape, elevation, and color) on Ziehl-Neelsen staining, then minimum inhibitory concentration was determined. The assessment was determined by researchers and expert lecturers.

Result

Extraction using *n*-butanol-methanol (1:1) produced brownish yellow colored extract filtrate that has an oil-like consistency and non-distinctive smell. The summary of results from the first week to the third week can be seen in table 1.

Table 1: Test results for antibacterial activity

No.	Materials	Lab. Code	Concentration (ppm)	Result Per Week			Descriptions
				1	2	3	
1	Extract	1	43.000	-	-	-	Inhibit the growth of *Mycobacterium tuberculosis* H37Rv (no growth)
		2	21.500	-	-	-	
		3	10.750	-	-	-	
		4	5.375	-	-	-	
		5	2.687,5	-	-	+	
		6	1.343,75	-	-	+	
		7	671,88	-	-	+	
		8	335,94	-	-	+	

Conted…

		9	167,97	-	-	+	Not inhibit the growth of *Mycobacterium tuberculosis* H37Rv (positive growth)
		10	83,98	-	-	+	
		11	41.99	-	-	+	
		12	21.00	-	-	+	
		13	10.50	-	+	+	
		14	5,25	-	+	+	
		15	2,62	-	+	+	
		16	1,31	-	+	+	
2	RIF		1,0	-	-	-	Inhibit the growth of *Mycobacterium tuberculosis* H37Rv (positive growth)
3	DMSO			-	-	+	Not inhibit the growth of *Mycobacterium tuberculosis* H37Rv (positive growth)
4	Control (+) M. tbH37Rv			-	+	+	

Note: (-): no growth (+) :Positive growth

On the first week, *Mycobacterium tuberculosis* H37Rv still has not shown any growth on Middlebrook 7H10 medium and Middlebrook 7H10 medium that contain solutions at several concentrations: 43.000 ppm, 21.500 ppm, 10.750 ppm, 5.375 ppm, 2.687,5 ppm, 1.343,75 ppm, 671,88 ppm, 335,94 ppm, 167,97 ppm, 83,98 ppm, 41,99 ppm, 21,00 ppm, 10,50 ppm, 5,25 ppm, 2,62 ppm, 1,31 ppm, likewise positive control (media and *Mycobacterium tuberculosis* H37Rv), Rifampicin (RIF) 1,0 ppm solution, and DMSO solvent. The growth of *Mycobacterium tuberculosis* H37Rv on Middlebrook 7H10 medium usually occur at the 1st-2nd week, so that observation continue for three weeks.

Observation at the second week shows that in a mastersolutionata concentration of 43.000 ppm to 21,00 ppm still has not shown the growth of *Mycobacterium tuberculosis* H37Rv. The solution at a concentration of 10,50 ppm to 1,31 ppm shows slight growth. The bacterial growth at a concentration of 10,50 ppm and 5,25 ppm can be seen as nebulous white dots, while at a concentration of 2,62 ppm the bacterial growth spread evenly as several small dots and at a concentration of 1,31 ppm show the most amount of growth compared to other concentrations. Positive control (media and *Mycobacterium tuberculosis* H37Rv) show a lot of growth with a white colored and slightly rough texture, in the Rifampicin (RIF) solution and DMSO still has not shown the bacterial growth. But it is not yet known whether the growth appears from *Mycobacterium tuberculosis* H37Rv or other bacteria.

At the third week, the inhibiting ability of extract *n*-butanol-methanol (1: 1) were decreasing. In a master solution at a concentration of 43.000 ppm to 5.375 ppm on Middlebrook 7H10 medium still has not shown the bacterial growth, which means at a concentration of 43.000 ppm, 21.500 ppm, 10.750 ppm, and 5.375 ppm has the inhibiting ability. Whereas at a concentration of 2.687,5 ppm to 1,31 ppm show bacterial growth. Since the first week to the third week, Rifampicin solution did not show any bacterial growth, which means Rifampicin as a standard that has the ability to inhibit *Mycobacterium tuberculosis* H37Rv growth. Whilepositive control (Middlebrook 7H10 medium and *Mycobacterium tuberculosis* H37Rv) in the second week to the third week show bacterial growth increment, this shows that *Mycobacterium tuberculosis* H37Rv can grow on selective Middlebrook 7H10 medium. But it was not yet known whether the growth appears from *Mycobacterium tuberculosis* H37Rv or other bacteria. Therefore Ziehl-Neelsen staining was undertaken to determine the bacteria that grow in each concentration.

Microscopic observation on Ziehl-Neelsen staining did not show *Mycobacterium tuberculosis* H37Rv at a concentration of 43.000 ppm; 21.500 ppm; 10.750 ppm; and 5.375 ppm, it was plain blue. Whereas at a concentration of 2.687,5 ppm; 1.343,75 ppm; 671,88 ppm; 335,94 ppm; 167,97 ppm; 83,98 ppm; 41,99 ppm; 21,00 ppm; 10,50 ppm; 5,25 ppm; 2,62 ppm; and 1,31 ppm show red, straight, slim rod shaped*Mycobacterium tuberculosis* H37Rv, with blue background.

To determine the bacterial activity in quantitative approaches, the lowest concentration that can inhibit bacterial growth was observed. At a concentration of 10.750 ppm has not shown the bacterial growth, which means this concentration has the ability to inhibit *Mycobacterium tuberculosis* H37Rv growth. At a concentration of 5,375 ppm also hasn't shown any bacterial growth, so this concentration still able to inhibit *Mycobacterium tuberculosis* H37Rv growth, but at a concentration of 2.687,5 ppm bacterial growth began to appear.

Microscopic observation on Ziehl-Neelsen staining did not show *Mycobacterium tuberculosis* H37Rv colony at a concentration of 43.000 ppm; 21.500 ppm; 10.750 ppm and 5.375 ppm, it was plain blue. Whereas at a concentration of 2.687,5 ppm; 1.343,75 ppm; 671,88 ppm; 335,94 ppm; 167,97 ppm; 83,98 ppm; 41,99 ppm; 21,00 ppm; 10,50 ppm; 5,25 ppm; 2,62 ppm and 1,31 ppm show red, straight, slim rod-shaped *Mycobacterium tuberculosis* H37Rv, with blue background. So that the Minimum Inhibitory Concentration (MIC) from extract *n*-butanol-methanol (1:1) filtrate solution of the fermentation results of *Streptomyces sp.* B10 against *Mycobacterium tuberculosis* H37Rv is 2.687,7 ppm. While the concentration of 5,375 ppm is the highest concentration that still able to kill the bacteria (MBC).

Table 2: Observation result of Minimum Inhibitory Concentration (MIC)

No.	Materials	Lab. Code	Concentration (ppm)	Result Per Week			Description
				1	2	3	
1	Extract	1	43.000	-	-	-	Inhibit the growth of *Mycobacterium tuberculosis* H37Rv (no growth)
		2	21.500	-	-	-	
		3	10.750	-	-	-	
		4	5.375	-	-	-	
		5	2.687,5	-	-	+	Not inhibit the growth of *Mycobacterium tuberculosis* H37Rv (appear growth)
		6	1.343,75	-	-	+	
		7	671,88	-	-	+	
		8	335,94	-	-	+	
		9	167,97	-	-	+	
		10	83,98	-	-	+	
		11	41.99	-	-	+	
		12	21.00	-	-	+	
		13	10.50	-	+	+	
		14	5,25	-	+	+	
		15	2,62	-	+	+	
		16	1,31	-	+	+	
2	RIF		1,0	-	-	-	Inhibit the growth of *Mycobacterium tuberculosis* H37Rv (no growth)
3	DMSO			-	-	+	Not inhibit the growth of *Mycobacterium tuberculosis* H37Rv (appear growth)
4	Control (+) M.tbH37Rv			-	+	+	

Note: (-) : no growth (+) : appear growth

The microscopic observation did not show *Mycobacterium tuberculosis* H37Rv colony at a concentration of 43.000 ppm; 21.500 ppm; 10.750 ppm and 5.375 ppm, it was plain blue. Whereas at a concentration of 2.687,5 ppm; 1.343,75 ppm; 671,88 ppm; 335,94 ppm; 167,97 ppm; 83,98 ppm; 41,99 ppm; 21,00 ppm; 10,50 ppm; 5,25 ppm; 2,62 ppm and 1,31 ppm show red, straight, slim rod-shaped *Mycobacterium tuberculosis* H37Rv, with blue background.

Discussion

Extract *n*-butanol-methanol (1:1) filtrate solution of the fermentation results of *Streptomyces sp.* B10 has antibacterial activity against *Mycobacterium tuberculosis* H37Rv. *n*-butanol is the selective solvent that can extract antibiotic compounds from *Streptomyces sp.* with a large zone of inhibition[15]. Ethanol extract has potent ability to inhibit bacterial growth, both gram-positive and gram-negative bacteria[17]. Methanol is a polar solvent that mostly used because of its efficient ability to penetrate into the cell wall, so it produces more endocellular secondary metabolites[16]. Several isozymesshows high substrate specificity and various homogeneity from *Streptomyces sp.* Cell extract for several types of alcohol[18].

Dilution using this extract can support the attack against *Mycobacterium tuberculosis* (TB). Since *Mycobacterium tuberculosis* has a high acquisition to drug resistance, new drug discovery to put into tuberculosis regimen is required[19]. *Streptomyces* potential for expressing foreign proteins indicate that *Streptomyces* can be a useful vector in designing new TB vaccine. Streptomycin is the first antibiotic that attacks*Mycobacterium tuberculosis* by mootherapy, so it leads to generate resistance. The mechanism of action of streptomycin is inhibiting mycobacterial protein synthesis in ribosomes. Resistance arises when mutations appear on rRNA and protein-encoding genes. ATCC27294 strains are sensitive toward the activity of streptomycin, rifampin, ethambutol, and isoniazid and ATCC35820 strains are resistant to streptomycin. Several mutated genes are evidenced to cause drug resistance[20,21]. The clinical trials of streptomycin show a good outlook to suppress TB. Although patient improved compared to the patient without therapy (considered as the first randomized controlled clinical trial), recurrence occurs in many patients and organisms are found to be resistant to streptomycin[22].

The concentration ranges from 2,687.5 ppm to 5,375 ppm effective against Mycobacterium. Antibacterial activity against *Mycobacterium tuberculosis* H37Rv occurs at a concentration of 2.687,5 ppm, which is determined as Minimum Inhibitory Concentration (MIC). Whilst the concentration of 5.375 ppm is determined as Minimal Bactericidal Concentration (MBC), which is the lowest concentration that able to kill bacteria. This result shows that antibacterial activity increased as the increasing extract *n*-butanol-methanol (1:1) filtrate solution of the fermentation results of *Streptomyces sp.* B10 concentration, because the extract in this study has not become an antibacterial pure compound.

There are few things asconsideration for further research: further analysis with separation of active compounds from Streptomyces sp. B10 using TLC bioautography method or other separation methods, further identification to find out another pure compound as antibacterial that produced by *Streptomyces sp.* B10, and limiting concentration range from 5.375 ppm to 2.687,5 ppm for antibacterial activity test and MIC determination.

Conclusion

Extract *n*-butanol-methanol (1:1) filtrate of the fermentation results of *Streptomyces sp.* B10 has antibacterial activity against *Mycobacterium tuberculosis* H37Rv. This indicates that this extract can be used as an alternative antibiotic formula to fight tuberculosis.

Ethical Clearance: This research has gone through ethical tests and permits from Faculty of Pharmacy Universitas Airlangga

Conflict of Interest: The author reports no conflict of interest of this work.

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