

Antituberculosis Activity of Brotowali (*Tinospora crispa*) Extract and Fractions against *Mycobacterium tuberculosis* using Microplate Alamar Blue Assay Method

Aktivitas Antituberkulosis Ekstrak dan Fraksi Batang Brotowali (*Tinospora crispa*) terhadap *Mycobacterium tuberculosis* dengan Metode Microplate Alamar Blue Assay

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ABSTRACT

Tuberculosis (TB), in which caused by pathogenic bacteria, *Mycobacterium tuberculosis*, has become the major causes of death among all of infectious diseases. The increasing incidence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) has created a need to discover a new antituberculosis drug candidate. The aim of this study was to screen extract and fractions of *Tinospora crispa* for activity against *Mycobacterium tuberculosis* H37Rv using the Microplate Alamar Blue Assay (MABA) method. *T. crispa* extract was prepared by maceration in ethanol (96%) and antituberculosis activity was carried out using MABA method. The result of this study showed that ethanolic extract of *T. crispa* exhibit antituberculosis activity with minimum inhibition concentration of 12.5 mg/ml.

ABSTRAK

Tuberkulosis (TB) adalah penyakit yang disebabkan oleh infeksi *Mycobacterium tuberculosis* dan menjadi penyakit infeksi yang menyebabkan paling banyak kematian di dunia. Daruratnya penyakit TB dan meningkatnya kasus multidrug-resistant tuberculosis (MDR-TB) dan XDR-TB ini mendorong dilakukannya banyak penelitian dan pengembangan obat anti-TB baru. Penelitian ini bertujuan untuk mengetahui aktivitas antituberkulosis batang Brotowali. Batang Brotowali kering diekstraksi dengan metode maserasi menggunakan pelarut etanol 96%. Uji aktivitas antituberkulosis secara *in vitro* terhadap *M. tuberculosis* H37Rv dilakukan dengan metode Microplate Alamar Blue Assay (MABA). Hasil penelitian menunjukkan bahwa ekstrak etanol batang Brotowali memiliki aktivitas antituberkulosis terhadap *M. tuberculosis* H37Rv dengan konsentrasi hambat minimum (KHM) sebesar 12,5 mg/ml.

INTRODUCTION

Tuberculosis (TB) is an infectious disease and the second leading cause of death from a single infectious disease in the world after human immunodeficiency virus (HIV) (Prabu *et al.*, 2015). In 2014, TB had caused 1.5 million of deaths (World Health Organization, 2015). It remains a major global health problem. This disease is caused by *Mycobacterium tuberculosis*, an intracellular obligate and aerobic bacillus that multiplies within macrophage.

TB treatment is generally consist of combination of four first line drugs: isoniazid, rifampicin, ethambutol, and pyrazinamide for two

months and followed by four additional months of isoniazid and rifampicin therapy (Nguta *et al.*, 2015). Unfortunately, in the last few decades, multi-drug resistant (MDR-TB) was arised. MDR-TB is caused by strain of *M. tuberculosis* that are resistant to isoniazid and rifampicin (Keshavjee and Farmer, 2012). In 2014, there were 480.000 MDR-TB cases and estimated 190.000 people died because of this disease (World Health Organization, 2015). Approximately 9.6% of the total MDR-TB cases are classified as extensively drug-resisten tuberculosis (XDR-TB) (Nguta *et al.*, 2015). In this cases, *M. tuberculosis* strain is not only resistant to isoniazid and rifampicin, but also resistant to any fluoroquinolones and to at least first line injectable antituberculosis drug, i.e amikacin, capreomycin, and kanamycin. The

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emergence of MDR-TB and followed by XDR-TB remains a major challenge to global health. This condition led to an urgent need to discover a new antituberculosis drug. Many researches are focused on the discovery and development of new antituberculosis drugs from natural product (Chinsebu, 2016). It can be done by screen novel compound from a natural product such as plant species (Alvin *et al.*, 2014). Natural products are considered as potential anti-TB agents (Nguta *et al.*, 2015)

Tinospora crispa (L.) Miers ex Hook.f.Thoms) is a member of Menispermaceae. It is widely known as Brotowali in Indonesia. It is widely distributed over tropical and subtropical Asia including India, Thailand, Vietnam, Philippines, Indonesia, and Malaysia (Chang *et al.*, 2015). This plant is commonly used as traditional medicine, especially its stem. The stem of *T. crispa* is traditionally used to treat tuberculosis. Some preliminary research reported the activity of *T. crispa* against *M. tuberculosis*. Ethanolic extract of *T. crispa* from Laos has antimycobacterial activity against *M. tuberculosis* with minimum inhibitory concentration (MIC) 2,43 – 96,2 µg/ml (Elkington *et al.*, 2014). But there has been no report about the antimycobacterial activity of *T. crispa* that grows in Indonesia and its fractions or isolates. The present study determined the antimycobacterial activity of ethanolic extract of *T. crispa* collected from Indonesia using microplate alamar blue assay (MABA) method.

METHODOLOGY

An antituberculosis activity using MABA assay was conducted in Tuberculosis Laboratory (BSL 2+), Faculty of Medicine, Universitas Gadjah Mada. Extraction and fractionation procedures were conducted in Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada.

Bacterial strain

M. tuberculosis H37Rv was obtained from Tuberculosis Laboratory, Faculty of Medicine, Universitas Gadjah Mada. This bacteria was subcultured on Lowenstein Jensen (LJ) medium. Suspension of log phase *M. tuberculosis* H37RV was prepared in 0.04% (v/v) Tween 80 and 0.2% bovine serum albumin (Sigma) so that their turbidities matches that of a McFarland no.1 turbidity standard. The turbidity was examined by weighing method (Kemenkes RI, 2012). Suspension was further diluted 1:25 in 7H9GC broth (4.7g of Middlebrook 7H9 broth base, 20mL of 10% glycerol, 880mL of distilled water, 100mL of OADC (oleic acid, albumin, dextrose, catalase)).

Chemicals and media

Middlebrook 7H9 broth (Difco), glycerol (Merck), OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson), Tween 80 (Sigma), Alamar Blue reagent (Invitrogen), sterile water, dimethyl sulfoxide, isoniazide (Fluka), rifampicin (Sigma), ethanol 96%, n-hexane, ethyl acetate, silika gel F₂₅₄ plate (Merck), ceric sulfate.

Plant material

Stems of *T. crispa* were collected in February 2016 from Sleman, Yogyakarta, Indonesia, and were authenticated in Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada.

Extraction

Dried and ground plant material (500 g) was extracted at room temperature by maceration method with ethanol 96% and repeated twice. Extraction process was done twice to get more effective extraction so that more compounds can be extracted. The extracts were then condensed using a Buchi rotary evaporator (rotavapor).

Fractionation

The extract was fractionated using trituration method. The ethanolic extract (5 g) was added with n-hexane and then shaken for 10 minutes. After shaking, it was filtered with sinterglass, then the filtrate were collected. The fractionation with n-hexane was conducted three times. The collected filtrate was condensed using a Buchi rotary evaporator (rotavapor) and it was called n-hexane fraction (F1). The residue, called n-hexane non-soluble fraction (F2), was then added with ethyl acetate and then shaken for 10 minutes. After shaking, it was filtered with sinterglass and the filtrate were collected. The fractionation with ethyl acetate was conducted three times. The collected filtrate condensed using a Buchi rotary evaporator (rotavapor) and called ethyl acetate fraction (F3). The residue was called ethyl acetate non-soluble fraction (F4).

Microplate Alamar Blue Assay (MABA) antituberculosis test

Antimycobacterial assay was perform using the Microplate Alamar blue assay (MABA) (Franzblau *et al.*, 1998). This assay was performed in 96-well microplates (Iwaki). Outer perimeter wells were filled with 200µL of sterile water to minimize evaporation of the medium in the experimental wells during incubation. One hundred microliters of 7H9GC broth were added to experimental wells. One hundred microliters of 100 mg/ml sample solution (extract

or fraction) were added to the wells in row B and C in columns 2, 3, and 4. By using micropipette, 100µL was transferred from row C to D and the contents of the wells were mixed well. This dilutions were continued through row G, and 100µL of excess medium was discarded from the wells in row G. Final extract concentration were 100; 50; 25; 12.5; 6.25 and 3.125 mg/ml (from row B to G). In the previous assay (not reported in this paper), we used final concentration ranges of 62.5 to 2000 µg/ml, but the result showed that it were not active. So, in the last assay, we used greater concentration of samples up to 100mg/mL. Row B to D columns 8 were used to isoniazide 10µg/mL as positive controls. Row B to D columns 9 were used to rifampicin 10µg/mL as positive controls. Row B to D columns 10 were used to dimethyl sulfoxide 4% as solvent controls. The wells in columns 11 served as sample-free (inoculum only) controls. One hundred microliters of *M. tuberculosis* suspension was added to the wells in row B to G in columns 2 to 11 yielding a final volume of 200µL per well. The plates were sealed with parafilm and were incubated at 37°C for 5 days. Starting at day 6 of incubation, 50µL of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to well B11 and plate were reincubated at 37°C for 24 h. If B11 became pink by 24 h, the reagent mixture was added to the entire plate (if the well remained blue, the reagent mixture would be added to another control well and the result would be read on the following day). The microplates were resealed with parafilm and were incubated for an additional 24 h at 37°C, and the colors of all well were recorded. A blue color in the well was interpreted as no growth and a pink color was scored as growth. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of samples which prevented a color change from blue to pink.

RESULTS AND DISCUSSION

T. crispa is a well-known source of herbal plant which is used traditionally as medications for diabetes mellitus, hypercholesterolemia and also hepatoprotective (Abu *et al.*, 2015). In order to screen the potentiality of *T. crispa* as antituberculosis candidate, ethanolic extract of the stem of *T. crispa* was tested against *M. tuberculosis* using Microplate Alamar Blue Assay (MABA).

MABA is a microplate method that used as high-throughput screen antituberculosis activity of samples. This method used Alamar blue, a resazurin-based oxidation-reduction based

indicator. This indicator delivered colorimetric MIC for *M. tuberculosis* in 14 days (Franzblau *et al.*, 1998). In this research, MIC for Ethanolic extract of Brotowali was available after 7 days of incubation. MABA is more effective and efficient than other method to determine drug or samples susceptibilities. *M. tuberculosis* is a slow growing mycobacteria and this method has significantly decrease the time to get the result. Biosafety cabinet, autoclave, and 37°C incubator are minimum major equipment to perform MABA.

Alamar blue dye is general indicator of celular growth and or viability; the blue and nonfluorescent. It is in blue color in the oxidized state, but it turns pink when reduced due to bacterial metabolism. Therefore, the growth of *M. tuberculosis* can be measured with fluorometer or spectrophotometer or determined by a visual color change (Collins and Franzblau, 1997). This is the major advantage of MABA. In this research, the growth of *M. tuberculosis* was determined by visual color change, without the use of specialized equipment. Collins and Franzblau (1997) reported that there were no statistically significant differences among MICs determined by fluorometric and visual MABA. The two colors (blue and pink) can be easily differentiated with the naked eye. Visual readout is the most frequently used in evaluation of antituberculosis compounds (Franzblau *et al.*, 2012).

MABA has a low possibility to resulting aerosols of *M. tuberculosis* suspension from well, so it can reduce the possibility of researcher may be infected during the assay. It is the other advantage of MABA method from biosafety perspective. H37Rv is a virulent strain of *M. tuberculosis*. This strain is still sensitive to rifampicin and isoniazide as antituberculosis drug used in tuberculosis therapy. H37Rv is the most strain used by laboratories in antituberculosis screening (Franzblau *et al.*, 2012).

Different culture media are in use for the isolation of mycobacteria. The most common is Lowenstein-Jensen (LJ) medium. It is an egg based medium with high concentrations of malachite green to overcome contamination with other bacteria. However, culture on it is long (3 weeks to 3 months for *M. tuberculosis*) and time consuming. In this research, we used Middlebrook 7H9 broth-based as the most media used in microplate-based assay. Glycerol was added as carbon source for culturing *M. tuberculosis*. While Tween 80 was used to minimize clumping of mycobacteria during growth.

Table I. Antimycobacterial activity results of *T. crisper* ethanolic extract and its fraction using MABA method

No	Sample name	Code	Concentration (mg/ml)	Result
1	Ethanolic extract of <i>T. crisper</i> stem	EEB	100	Active
			50	Active
			25	Active
			12.5	Active
			6.25	Not active
			3.125	Not active
			2	Not active
2	n-hexane soluble fraction	F1	1	Not active
			0.5	Not active
			0.25	Not active
			0.125	Not active
			0.0625	Not active
			2	Not active
			1	Not active
3	n-hexane non soluble fraction	F2	0.5	Not active
			0.25	Not active
			0.125	Not active
			0.0625	Not active
			2	Not active
			1	Not active
			0.5	Not active
4	Ethyl acetate soluble fraction	F3	0.25	Not active
			0.125	Not active
			0.0625	Not active
			2	Not active
			1	Not active
			0.5	Not active
			0.25	Not active
5	Ethyl acetate non soluble fraction	F4	0.125	Not active
			0.0625	Not active
			0.01	Active
			0.01	Active
6	Isoniazid 10 µg/ml	K+	0.01	Active
7	Rifampisin 10 µg/ml	K+	0.01	Active
8	DMSO 4%	Kp		Not active

Sample was categorized as active if it can prevent a color change from blue to pink. In this study, we tested extract and fraction of *T. crisper* with 100 mg/ml as the highest concentration because it were not active under 100 µg/ml. Most paper suggested that plants extract considered had significant antituberculosis activity if MIC was < 100µg/mL. There were many different reference to analyze the antituberculosis activity of plant extracts. In previous study, plants extract were considered active against *M. tuberculosis* if MIC was <100µg/mL (Borges-Argáez *et al.*, 2007; Gautam *et al.*, 2007; Okunade *et al.*, 2004), <200µg/mL (Camacho-Corona *et al.*, 2008; Tosun *et al.*, 2004), < 1600µg/mL (Mohamad *et al.*, 2011), ≤ 2048 µg/ml (Tekwu *et al.*, 2012). In other

paper, the antimicrobial activity of plant extracts was classified as significant (MIC ≤100µg/mL), moderate (100 < MIC < 625µg/mL), or weak (MIC > 625 µg/mL) (Kuate, 2010). Because of ethanolic extract of Brotowali exhibited antituberculosis activity against *M. tuberculosis* H37Rv with MIC 12.5mg/mL, so we interpreted that this activity was weak.

The results of antituberculosis assay of extract and its fractions against *M. tuberculosis* H37Rv are shown in Table 1. These results showed that from *T. crisper* extract and fractions tested, ethanolic extract of Brotowali exhibited antituberculosis activity against *M. tuberculosis* H37Rv with MIC 12.5mg/mL. This was contradictive with the result of previous research

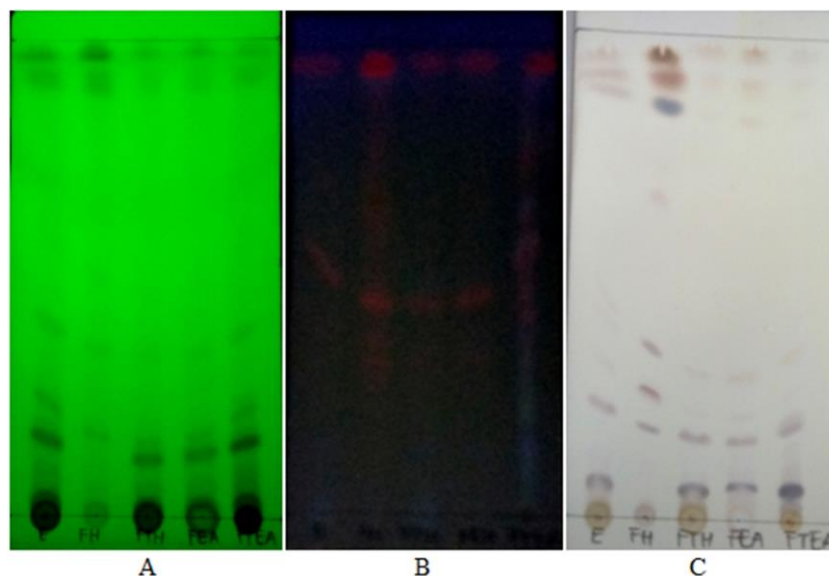


Figure 1. Thin layer chromatography profile of Brotowali ethanolic extract and its fraction on silica gel 60 F₂₅₄ plate as stationary phase, and chloroform:methanol (9:1) as mobile phase. A : under uv 254 nm detection; B : under uv 366 nm detection; C : after sprayed by ceric sulfate.

that ethanolic extract of *T. crispa* from Laos has antimycobacterial activity against *M. tuberculosis* with minimum inhibitory concentration (MIC) 2,43-96,2µg/mL. The difference of plant materials collection location could be one of the reason of this different results. Different type of soil, different organic component in soil can influence the secondary metabolite contains in plant. This can affect the biological and pharmacology activity of the plant.

The fractions (F1, F2, F3, and F4) were result of separation procedures of *T. crispa* extract. This separation procedures were conducted to separate the compound content in the extract based on its polarity properties. n-hexane fraction (F1) contains non polar compounds, n-hexane non-soluble fraction contains compounds that its more polar than F1. Ethyl acetate fraction (F3) contains semi-polar compounds and ethyl acetate non-soluble fraction (F4) contains polar compounds. So, if the result of antituberculosis assay of fractions were active and potential to be developed as new antituberculosis drug candidates, it will become easier to isolate the active compound from the active fraction. Unfortunately, all fractions did not exhibited antituberculosis activity against *M.tuberculosis* H37Rv.

Based on chromatogram on figure 1, Brotowali ethanolic extract exhibited a clear

separation using chloroform and methanol as mobile phase. n-hexane soluble fraction (F1) contains non-polar compounds dominantly, while the polar compounds contained in the ethyl acetate non soluble fraction (F4). *T. crispa* contains a wide range of secondary metabolites such as alkaloids, terpens, flavons and phenolics (Koay and Koay, 2013). This compounds exhibited antitubercular activity (Okunade, *et al.*, 2004; Garcia *et al.*, 2012). But, in this research, we did not do any test to find out which compounds in *T. crispa* extract had the responsibility of the antituberculosis activity.

CONCLUSIONS

In conclusion, our data show that ethanolic extract of *T. crispa* has weak antituberculosis activity against *M. tuberculosis* with MIC of 12.5 mg/ml.

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