ANTITUBERCULOSIS ACTIVITY OF EXTRACT AND FRACTIONS OF *TINOSPORA CRISPA* AGAINST *MYCOBACTERIUM TUBERCULOSIS* H37RV USING MYCOBACTERIA GROWTH INDICATOR TUBE AND AGAR PROPORTION METHOD

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ABSTRACT

Objective: The increasing incidence of multidrug-resistant tuberculosis (TB) has created a need to discover a new anti-TB drug candidates. The aim of this study was to screen extract and fractions of *Tinospora crispa* for activity against *Mycobacterium tuberculosis* H37Rv.

Methods: The dried and pulverized *T. crispa* stem was extracted by maceration method using ethanol (96%). The anti-TB activity was carried out using mycobacteria growth indicator tube (MGIT) system and agar proportion method with Lowenstein–Jensen (LJ) medium.

Result: The result of this study showed that ethanolic extract and fractions of *T. Crispa* did not exhibit anti-TB activity in the range of 100–1000 μg/ml with MGIT method, while with agar proportion method, there were *M. tuberculosis* colonies growth on the LJ containing 1000 μg/ml extract slants.

Conclusion: The tested extract and fractions of *T. crispa* have no anti-TB activity against *M. tuberculosis* until 1000 μg/ml.

Keywords: *Mycobacterium tuberculosis, Tinospora crispa*, Tuberculosis activity.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that causes most widely death in the world [1]. In 2014, TB caused 1.5 million of deaths [2]. It remains a major global health problem. This disease is caused by *Mycobacterium tuberculosis*, an intracellular obligate and aerobic bacillus that multiplies within macrophage. *M. tuberculosis* infects the lungs and can spread to other human through droplets from the throat of people-infected TB. Beside of the lungs, this bacteria can affect other organs in the human body such as lymph gland, abdomen, skin, joints, and meninges.

TB occurs in every part of the world. In low- and middle-income countries, TB cases occurred over 95% [3]. About 58% of reported TB cases in the world occurred in Southeast Asia and West Pacific [2]. Indonesia becomes the second country in the world with the largest TB cases. In the global TB report of the year 2015, the WHO informed that there were 9.6 million TB cases and 12% of new cases were coinfected with HIV.

TB is a curable disease with a combination of four first-line drugs: Isoniazid, rifampicin, pyrazinamide, and ethambutol, for a regimen of at least 6–9 months. Unfortunately, in the past few decades, multidrug-resistant TB (MDR-TB) was arised. MDR-TB is caused by strain of *M. tuberculosis* that is resistant to isoniazid and rifampicin [4]. The resistance is caused by mutation in the nucleic acid of the bacteria. For assessing bacterial resistance, molecular detection technique is known fast and specific [5]. In 2014, there were 480.000 MDR-TB cases and estimated 190.000 people are died because of this disease [2]. Patients' adherence to TB treatment is important to get the effective treatment and to prevent the occurrence of multidrug-resistant MDR-TB [6]. Approximately 10% of the total MDR-TB cases are classified as extensively drug-resistant TB (XDR-TB) [7]. In these 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 anti-TB drug, i.e, amikacin, capreomycin, and kanamycin. The 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 new anti-TB drugs. Many research is focused on the discovery and development of new anti-TB drug from natural product [1]. The discovery of new anti-TB drug candidates who are more effective against MDR-TB strain and XDR-TB strain became very urgent, especially in developing countries with high incidence of TB. It can be done by screen from novel compound from natural product including plant species [8]. Medicinal plants can be potential anti-TB drug candidate if it is proven by scientific studies first. Some of them used as food ingredient, and it will be more efficient to minimize the disease [9]. Not only plants but also other natural product can be used as a source of new anti-TB agents. Crude extract from a *Bacillus* sp. N strain associated with entomopathogenic nematode *Rhabditis* (*Oscheius*) sp. has antimycobacterial activity against *M. tuberculosis* H37Rv with a MIC of 125 μg/ml [10].

Tinospora crispa (L.) Miers ex Hook. f. Thoms is a member of Menispermaceae. It is popularly known as Brotowali in Indonesia. It is widely distributed over tropical and subtropical Asia including India, Thailand, Vietnam, Philippines, Indonesia, and Malaysia [11]. This plant is widely used as traditional medicine, especially its stem. The stem of *T. crispa* is traditionally used to treat TB. 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 [12]. However, 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 and fractions of *T. crispa* collected from Indonesia.

MATERIALS AND METHODS

Materials

The stem of *T. crispa* was collected in February 2016 from Sleman, Yogyakarta, Indonesia, and was authenticated in the Department of

Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University. The stem was dried at 40°C in a drying cabinet and was ground with grinding machine before extraction. Each sample was dissolved in dimethyl sulfoxide 4% (Sigma–Aldrich Chemical). *M. tuberculosis* H37Rv used in this study was collected from Balai Besar Laboratorium Kesehatan, East Java (Indonesia). Middlebrook (MB) 7H9 broth (Difco), oleic acid/albumin/dextrose/catalase enrichment (OADC) (Becton–Dickinson and Company), Polymixin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin (Becton–Dickinson and Company), Tween 80 (Sigma–Aldrich Chemical), Isoniazid (Sigma–Aldrich Chemical), Rifampicin (Sigma–Aldrich Chemical), and Kit MGIT BD Biosciences (Becton–Dickinson Company) were used. Lowenstein–Jensen (LJ) medium was prepared following the standard operating procedures recommended by the Indonesian National TB Program [13], which are in line with the WHO guidelines [14].

Extraction
Dried and ground plant material (500 g) was extracted by maceration method with ethanol 96% and repeated twice. The extracts were then condensed using a Buchi rotary evaporator (rotavapor).

Fractionation
Dried and ground plant material (500 g) was extracted by maceration method with ethanol 96% and repeated twice. The extracts were then condensed using a Buchi rotary evaporator (rotavapor) yielded 136.6 g thick extract. The extract was fractionated using trituration method. The ethanolic extract (25 g) added with n-hexane and then shaked for 10 min. After shaking, it filtered with Sinterglass, and then, the filtrate was collected. The fractionation with n-hexane was conducted 3 times. The collected filtrate condensed using a rotavapor and yielded n-hexane fraction, FH (10 g). The residue was called n-hexane-insoluble fraction, FTH (15 g). Five gram (5 g) of FTH then added with ethyl acetate and then shaked for 10 min. After shaking, it filtered with Sinterglass, and then, the filtrate was collected. The fractionation with ethyl acetate was conducted 3 times. The collected filtrate condensed using a rotavapor and its called ethyl acetate fraction, FEA (2.2 g). The residue was called ethyl acetate-insoluble fraction, FTEA (2.8 g).

MGIT anti-TB test
In this study, *M. tuberculosis* H37Rv was used. *M. tuberculosis* was cultured on the mycobacteria growth indicator tube (MGIT) with BACTEC MGIT 960 growth supplement. MGIT 960 instrument (Becton Dickinson Diagnostic Systems, Sparks, Maryland, USA) system was used in this study to evaluate antimycobacterial activity of extract and its fractions against *M. tuberculosis* H37Rv. Before inoculating with the bacteria, MGIT growth tubes containing 7 ml media (BBL™ MGIT™ Bactec and Dickinson, USA) were supplemented with MB growth supplement (Oleic acid, Albumin, Dextrose, Catalase; OADC) as specified in the manufacturer's protocol (800 µl/tube). After adding the growth supplement, 100 µl aliquots of extract or fraction were added aseptically

into the MGIT tubes at different concentrations. For inoculation, the bacterial stock was thawed and prepared in 7H9-S media so that the inoculation volume (500 µl) contained sufficient numbers of bacterial cell ($1×10^5$ cfu/ml in a tube). The contents were mixed gently by inverting the tube 3–4 times. All inoculated tubes were then inserted into the MGIT 960 automated culture system after scanning the barcode and incubated at the temperature of 37°C. For positive control, isoniazid, rifampicin, or no drugs were used. The growth control (GC) tube was prepared by adding 800 µl growth supplement and 500 µl bacterial suspension into MGIT tube. The GC did not contain any samples. In the case of positive growth, the system automatically detects growth and signals positive. After a maximum of 6 weeks (42 days), the instrument flags a tube as negative if no growth occurs. The results are qualitative such as susceptible (S), resistant (R), or indeterminate (X). The instrument interprets results at the time when the growth unit (GU) in GC reaches 400. At this point, the GU values of the drug vial are evaluated: Susceptible when the GU of the drug tube is <100 and resistant when the GU of the drug tube is 100 or more. Further, X results when certain conditions occur which may affect the test, such as GU of the control reaches >400 in <4 days [15].

Anti-TB test with agar proportion method
Anti-TB test with agar proportion method using LJ media was carried out to confirm the MGIT result. For this purpose, 1 ml of extract and 100 µl of *M. tuberculosis* H37Rv suspension were added into MB 7H9. After 2 days of incubation, 100 µl of the mixture was subcultured onto LJ slants. Then, it was incubated for 4–6 weeks at 37°C. The growth of the mycobacterial colonies (rough, tough, and buff-colored) was inspected. The mycobacterial colonies on the LJ slants indicated that the extract cannot inhibit the mycobacterial growth.

RESULTS

The results of anti-TB assay of extract and its fractions against *M. tuberculosis* H37Rv are shown in Table 1. These results showed that from *T. crispa* extract and fractions tested, none of them exhibited anti-TB activity against *M. tuberculosis* H37Rv up to 1000 µg/ml. This was contradictive with the result of previous research that ethanolic extract of *T. crispa* from Laos has antimycobacterial activity against *M. tuberculosis* with MIC 2.43–96.2 µg/ml. The antimycobacterial activity that used in the research was microplate Alamar blue assay. The difference of method used in this research and previous research could be one of the reasons for this different results. Time for obtaining results was 9 days.

To confirm the MGIT results, anti-TB activity of *T. crispa* ethanolic extract was tested with agar proportion method. There were 3 concentrations tested: 1000, 500, and 250 µg/ml. There was *M. tuberculosis* colony growth on the LJ containing extract slants. It was compared to *M. tuberculosis* slant (K+) and they showed the same appearance of

Table 1: Antimycobacterial activity results of *T. crispa* ethanolic extract and its fraction using MGIT system

No	Sample	Code	Concentration (µg/ml)	Growth unit	Result
1	Ethanolic extract of *T. crispa* stem	EEB	1000	400	Not active
			500	400	Not active
			100	400	Not active
2	n-hexane-soluble fraction	FH	1000	400	Not active
			500	400	Not active
			100	400	Not active
3	n-hexane-insoluble fraction	FTH	1000	400	Not active
			500	400	Not active
			100	400	Not active
4	Ethyl acetate-soluble fraction	FEA	1000	400	Not active
			500	400	Not active
			100	400	Not active
5	Ethyl acetate-insoluble fraction	FTEA	1000	400	Not active
			500	400	Not active
			100	400	Not active

T. crispa: Tinospora crispa, MGIT: Mycobacteria growth indicator tube

the slants. As shown in Fig. 1, the positive control slant which contains rifampicin as anti-TB drug showed that there are no yellow colonies of *M. tuberculosis*. The results of this test showed that *T. crispa* ethanolic extract up to 1000 µg/ml did not exhibited anti-TB activity.

DISCUSSION

T. crispa is a well-known source of herbal plant which is used traditionally as medications for diabetes mellitus, hypercholesterolemia, and also hepatoprotective [16]. Ethanolic extract of *T. crispa* stem showed general standardization parameters. This plant has the potential compounds to be developed as a raw material of standardized herbal medicine [17]. To screen the potentiality of *T. crispa* as anti-TB candidate, ethanolic extract of the stem of *T. crispa* was tested against *M. tuberculosis* using MGIT system. Bactec MGIT 960 - an automated liquid system - has been developed to reduce time of experiment [18]. It is much shorter than agar proportion method (turnaround time is around 21 days). MGIT 960 system is a reliable, rapid, automated method for testing the susceptibility of clinical *M. tuberculosis* isolates to the first-line drugs [19]. Time for obtaining results in this research was 9 days, and it is longer than that observed by Kontos *et al.* (2004). It needed 7.12 days to obtain the MGIT results.

Different culture media are used for the isolation of mycobacteria. The most common is LJ medium, an egg-based medium. LJ medium contains high concentrations of malachite green to prevent contamination with other bacteria. However, culture on it is long (3 weeks to 3 months for *M. tuberculosis*) and time-consuming. MGIT was better than LJ medium in recovery rate and detection time of mycobacterial growth [20]. Due to its sensitivity, MGIT should be used for smear-negative pulmonary TB patients [21]. MGIT system could be an acceptable alternative to the radiometric Bactec method for rapid and reliable testing of *M. tuberculosis* [22].

The MGIT consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains modified Middlebrook 7H9 broth base. The MGIT tube contains an oxygen-quenched fluorochrome, tris 4,7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is used and is replaced with carbon dioxide. The decrease of free oxygen made the fluorochrome was no longer inhibited and produces a fluorescence within the MGIT tube when visualized under UV light [20].

The relative growth ratio between the sample-containing tube and sample-free GC tube was determined by the system's software algorithm, once the GC became positive. If the relative growth of the sample containing tube was equal to or exceeded that of the GC tube, the bacterial was considered resistant; if the relative growth was less than in the GC tube, the bacterial was considered susceptible. The instrument did the final interpretation and reported the result automatically.
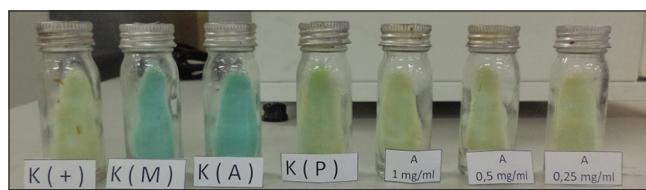

Fig. 1: *Tinospora crispa* ethanolic extract which has been inoculated with *Mycobacterium tuberculosis* on Lowenstein–Jensen medium slant at 37°C for 4 weeks. (a) *M. tuberculosis* control; (b) medium control (without *M. tuberculosis* suspension); (c) positive control, rifampicin 40 µg/ml; (d) solvent control (dimethyl sulfoxide 5%); (e) *T. crispa* ethanolic extract of concentration 1000 µg/ml; (f) *T. crispa* ethanolic extract of concentration 500 µg/ml; (g) *T. crispa* ethanolic extract of concentration 250 µg/ml

There are many different references to analyze the anti-TB activity of plant extracts. In the previous study, plant extract was considered active against *M. tuberculosis* if MIC was <100 µg/ml [23-25], <200 µg/ml [26,27], <1600 µg/ml [28], and ≤2048 µg/ml [29]. In other paper, the antimicrobial activity of plant extracts was classified as significant (MIC ≤100 µg/ml), moderate (100< MIC <625 µg/ml), or weak (MIC >625 µg/ml) [30]. In this study, we tested extract and fraction of *T. crispa* at three concentration with 1000 µg/ml as the highest concentration. Hence, we interpreted activity as inhibition at any values of MIC ≤1000 µg/ml.

Agar proportion method with LJ medium was conducted due to confirm the results of anti-TB activity test with MGIT method. This method is the current standard method used in Indonesia for drug susceptibility testing of *M. tuberculosis* against anti-TB drugs. It is required at least 28 days for obtaining the results, and this is the drawback of this method. Mycobacterial growth on cultures was considered as positive as soon as yellow colonies appear on LJ medium. In this test, we used three concentration series, i.e., 1000, 500, and 250 µg/ml. The results of this test showed that *T. crispa* ethanolic extract up to 1000 µg/ml did not exhibited anti-TB activity. This results obtained an agreement between the MGIT method and the agar proportion method with LJ medium.

Ethanolic extract of Brotowali stem contains flavonoid as a group of compounds that have antiradical activity. It has DPPH radical scavenging activity with IC_{50} was 33.75 µg/ml [31]. *T. crispa* contains a wide range of secondary metabolites such as alkaloids, terpenes, flavones, and phenolics [32]. This compounds exhibited antitubercular activity [25,33]. However, in this research, we did not do any test to find what compounds are in *T. crispa* extract and fractions.

CONCLUSIONS

Our data show that ethanolic extract and fractions of *T. crispa* have no anti-TB activity against *M. tuberculosis* until 1000 µg/ml.

AUTHORS CONTRIBUTION

All the author have contributed equally.

CONFLICTS OF INTERESTS

We have no conflict of interest to declare

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