ORIGINAL ARTICLE

The ethanolic extract of ashitaba stem (*Angelica keskei* [Miq.] Koidz) as future antituberculosis

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ABSTRACT

Considering the easy contagion of tuberculosis (TB) disease spread and the emergence of multidrug-resistant TB, which directly impacts the failure of therapeutic goals and mortality rates increasing, TB disease control remains to be the main concern of continuous health development effort. Therefore, the discovery of new TB drug is needed. This research assessed the new natural anti-TB drug from the ethanolic extract of *Angelica keiskei* stem obtained from Lombok, Indonesia. The objectives of this study were to evaluate the sensitivity of *Mycobacterium tuberculosis* (Mtb) H37Rv strain to *A. keiskei* stem extract and to determine its minimum inhibitory concentration (MIC). The extraction methods of *A. keiskei* stem were done using a maceration method. In addition to phytochemical screening and water content analysis using standard method, the phytochemical parameters were analyzed by thin-layer chromatography. Ethanolic extract of *A. keiskei* stem was assayed for their Mtb inhibitory activity using the proportion method. The phytochemical analysis result showed that the secondary metabolites contain in the extract were flavonoid, polyphenol, tannin, monoterpenoid and sesquiterpen, quinon, and saponin. The anti-TB test result showed the active activity of ethanolic extract of *A. keiskei* against Mtb H37Rv strain with MIC ranging from 6% to 8% w/v. In conclusion, ethanolic extract of *A. keiskei* is a prospective natural anti-TB for the future.

Key words: *Angelica keiskei,* Indonesia, minimum inhibitory concentration, *Mycobacterium tuberculosis* H37Rv, tuberculosis

INTRODUCTION

Tuberculosis (TB) is the world's number one killer among other infectious diseases, caused by *Mycobacterium tuberculosis* (Mtb), which is an intracellular facultative bacillus. TB has been around for thousands of years and is still a major global health problem. TB is one among the 10 causes of death worldwide. There are an estimated 10.4 million new TB cases worldwide, of which 5.9 million (56%) are male, 3.5 million (34%) are female, and 1.0 million (10%) of them are children.[1] However, control of TB transmission has been developing for many years worldwide, especially in developing countries. In Indonesia, the government made a strategy by giving free medication and treatment for TB patient. However, the eradication is still facing many problems because the long-term treatment of TB is very influential on patient compliance. TB patients need 2 months of intensive phase therapy followed by 4 months of maintenance phase therapy.[2]

In addition, Mtb developed resistance against both the first-line and the second-line drugs.[3] Another study

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reported that 6800 new cases of multidrug-resistant TB (MDR-TB) were developed in Indonesia and >55% of MDR-TB patients were not correctly diagnosed or treated.[4] The resistant strain spread would complicate TB treatment. Therefore, several studies have been screening new anti-TB from plant, to reduce side effect of existing antibiotics therapy and alternative treatment for Mtb-resistant strain.

Some natural products and their derivatives have been reported to exhibit extraordinary growth inhibitory activity against Mtb, and some of them have even been selected as prototype molecules for the development of new anti-TB agents.[5,6] Of plant extracts, antimycobacterial compounds with the mechanism of activity have been reported.[7,8] *Angelica keiskei* Koidzumi, or ashitaba, is a popular botanical medicine containing diverse bioactive components, including prenylated chalcones, linear and angular coumarins, and flavanones. Due to those metabolite contents, *A. keiskei* has been reported to show antimicrobial activity.[9] The chalcones were one of the compounds that had been exhibited the anti-TB activity.[10] Besides the activity, *A. keiskei* also has been demonstrated *in vivo* and the result is not toxic.[11] Therefore, the objectives of this study were to evaluate the sensitivity of Mtb H37Rv strain to *A. keiskei* stems extract and to determine its minimum inhibitory concentration (MIC).

MATERIALS AND METHODS

Plant materials

The plant material used in this research is ashitaba stem (*A. keiskei* [Miq.] Koidz). The family name of this plant is *Apiaceae*. The plant was taken from a plantation in Lombok, West Nusa Tenggara. Ashitaba grows well in upland areas with deep moist soil as in Sembalun, Lombok. After 1 month or diameter of the ashitaba stem reaches 0.8–1.0 cm, the crop was harvested and identified at a Research Centre For Biology, Indonesian Institute of Science, Cibinong, Indonesia.

Mycobacterium strain

Mtb H37Rv ATCC 27294 was obtained from the Health Laboratory Department in Bandung, Indonesia.

Extraction

The ashitaba stems were cleaned after harvest using water and dried at room temperature. A total of 500 g of fresh ashitaba stems was dried and mashed to a small flake using paper scissors and then macerated successively in 70% ethanol for 3 × 24 h. The macerates were then filtered and concentrated with a rotavapor at 40°C. The water content of the extract then analyzed by distillation method with toluene solvent.

Phytochemical screening

Phytochemical screening was performed on the simplicia and ashitaba extracts to determine their chemical content, such as alkaloids, tannins and polyphenol, flavonoids, monoterpenoids, sesquiterpenoids, steroids, triterpenoids, quinones, and saponins. The phytochemical screening method was performed according to the standard methods.[12]

Antituberculosis activity test

Antibacterial activity of ashitaba stem extract against Mtb *H37rv* was done using proportion method. The standard methods using Löwenstein–Jensen (LJ) medium includes the proportion method, absolute concentration method, and resistant ratio method, which are fairly well standardized for the major anti-TB drugs.[13] Besides the extract, drug susceptibility of Mtb was also determined by the observation of Mtb growth on the surface of LJ medium containing rifampicin, ethambutol, streptomycin, and isoniazid as positive controls. The determination of colony forming units (cfu) on LJ medium was performed by dilution 10 times from the standard suspension of 1 mg/ml Mtb and disseminated in LJ test medium. Each of LJ bottle, containing extracts, antibiotic (as positive controls), and negative control (without extract), was diluted to 10^{-3} and 10^{-5} in bacterial suspension 10^{7}–10^{8} cfu/ml. The dilution results were inoculated into LJ tube controls and to each tube that containing 4 mg/L streptomycin, 0.2 or 1 mg/L isoniazid, 40 mg/L rifampicin, and 2 mg/L ethambutol. All media were incubated at 37°C and colonies calculations were calculated after 21, 28, and 42 days. The proportion of resistant mycobacteria was calculated as the number of colonies grown in the tube containing the extract as compared to the control.

Mycobacterium sensitivity test on ashitaba extract

The sensitivity test was done using Middlebrook 7H9 broth medium in BacT/ALERT 3D system.[14] Exposure of the mycobacterial suspension (0.2 ml, 1 mg/ml) to millipore (0.22 μm), then 4% v/v ashitaba stem extract was filtered and mixed homogeneously for 15 min at room temperature. The mixture was inoculated in a mycobacterial process (MP) bottle containing the Middlebrook 7H9 broth supplemented with reconstitution fluid (oleic acid, glycerol, and bovine albumin serum) in the BacT/ALERT 3D colorimetric system (BioMerieux, France). The bottles were incubated at 37°C. In accordance with the method used to establish resistance to anti-TB drugs, a relative delay of 3.5 days in a positive drug (ashitaba extract) in which the bottle contains free control extract is considered as the criterion for resistance to the medium containing the extract. Considered to be susceptible to extract (there is a growth observation) if the bottle containing the extract is marked with no positive after 3.5 days on the device obtained from a positive signal in the control of the over-the-counter drug. This is equivalent to over 90% of the inhibition of mycobacteria by the extract (as an antimicrobial agent) compared to the medium with no addition of the extract. The BACTEC

system (Becton-Dickinson, Sparks, MD) uses a liquid medium and detects mycobacteria based on the tracker that will detect CO_2 release. The advantages of this BACTEC system include shorter incubation (9–14 days), possibility of degradable drugs in smaller mediums, and when some concentrations are tested, it will result in a quantitative endpoint (MIC).

RESULTS

Extraction results

The extraction process resulted extract rendemen in 27.52% from 500 g of ashitaba stem simplicia. From the examination of moisture content, water content obtained was 1%.

Phytochemical-screening results

Based on the results of phytochemical screening of simplicia and extract, it can be concluded that ashitaba stems have flavonoid compounds, polyphenols, tannins, monoterpenoids and sesquiterpenes, quinones, and saponins. This is in accordance with another study that the stem ashitaba contains the same metabolites.[15]

Sensitivity test results

Sensitivity test of Mtb strain of H37Rv on stem extract of ashitaba was done by the proportion method using a variation of concentration of 10%, 20%, 30%, 40%, and 50% w/v. The results could be seen in Table 1.

Result of minimal inhibitory concentration determination

Based on the MIC test results presented in Table 2, ashitaba stem extract started to provide inhibition to the growth of *M. tuberculosis* at a concentration of 6% w/v. This could be seen from the decreasing amount of colonies from medium containing 8% w/v extract concentration of media with an extract concentration of 6% w/v. Thus, it could be concluded that the MIC of ashitaba ethanol extract was ranging in concentration of 6–8% w/v.

Interpretation result of sensitization determination using the proportion method

The result of this method demonstrated that percentage of Mtb proportions with extracts concentrations of 10%, 20%, 30%, 40%, and 50% w/v in 10^{-3} and 10^{-5} bacterial concentrations on day 28 and 42 were below 1%. This suggested that the Mtb strain H37Rv was sensitive to the ethanol extract of ashitaba stem (*A. keiskei* K.). Mtb strain of H37Rv is said to have a sensitivity when the percentage value of the proportion is below 1%.[16] Thus, it can be concluded Mtb strain H37Rv had a sensitivity to ethanol extract of ashitaba stem.

DISCUSSION

Based on the data in Tables 1 and 3, it was known that the Mtb strain H37Rv sensitive to variations in the concentration

Table 1: Sensitivity test results

Agents	Concentration (%v/v)	Bacterial suspension	Colonies growth (weeks)					
			1	2	3	4	5	6
Extract ashitaba	50	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	40	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	30	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	20	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	10	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
Antituberculosis drug	Rifampicin	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	Isoniazid	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	Streptomycin	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
	Ethambutol	10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	-
Positive control		10^{-3}	-	1+	1+	1+	1+	1+
		10^{-5}	8	10	10	10	10	
Negative control		10^{-3}	-	-	-	-	-	-
		10^{-5}	-	-	-	-	-	

-: Absence, +: Presence

of the ashitaba ethanol extract. It was characterized by the absence of bacterial colony growth at each concentration of the extract. In addition, Mtb strain H37Rv was sensitive to the first-line antituberculous drugs. Several herbs have been reported to possess antituberculous activity similar to the current study.[17,18] Some of these secondary metabolites such as alkaloids, polyphenols, flavonoids, and terpenoids are known to be potential antitubercular drugs. The mechanism of action of flavonoids and some phenolic compounds as antimicrobacterial by destroying cytoplasmic membranes with perforation inhibits nucleic acid synthesis, disrupts energy metabolism by inhibiting nicotinamide adenine dinucleotide plus hydrogen-cytochrome c reductase, and destroys cytoplasmic membranes by producing hydrogen peroxide, inhibiting ATP synthase, and inhibiting catalytic activity of DNA topoisomerase I and II.[19] From a number of plants, alkaloids have been reported to perform effectively against Mtb.[17] Alkaloid vasicine from *Adhatoda vasica* and aristolactam-type alkaloid from *Aristolochia brevipes* have been confirmed to possess antimycobacterial activity.[20,21] Plant terpenoids have already reported to show antimycobacterial activity.[22,23] Flavonoids are another important class of phytochemicals possessing antimycobacterial activity.[23] Although sometimes, phytochemicals are not as effective as synthetic drugs, it can provide a synergistic effect in combinations for inhibiting pathogens.[24] As reported in another study, that combination of rifampicin and ethanolic extracts of *Hibiscus sabdariffa* calyces, *Kaempferia galanga* rhizomes, and *Piper crocatum crocatum* leaves achieved good combination effects against the rifampicin/streptomycin-resistant strain.[25] In addition, the first-line antituberculous drugs are administered for a long period (6–8 months). This could develop resistance cases and the failure of TB treatment because of patient compliance. Therefore, phytochemicals may become the base for new drug development by providing a pharmacophore which could be used for the development of new drug with novel mechanism of action.[23]

Table 2: Minimum inhibitory concentration determination results

Concentration (%w/v)	Bacterial suspension	Colonies growth (weeks)					
		1	2	3	4	5	6
8	10^{-3}	-	-	-	-	-	-
	10^{-5}	-	-	-	-	-	-
6	10^{-3}	-	-	-	-	4	17
	10^{-5}	-	-	-	-	-	-
4	10^{-3}	-	-	2	12	1+	1+
	10^{-5}	-	-	-	-	4	6
2	10^{-3}	-	3	1+	1+	1+	1+
	10^{-5}	-	-	1+	1+	1+	1+
1	10^{-3}	-	5	1+	2+	2+	2+
	10^{-5}	-	-	11	16	16	16
Streptomycin	10^{-3}	-	-	-	-	-	-
	10^{-5}	-	-	-	-	-	-
Isoniazid	10^{-3}	-	-	-	-	-	-
	10^{-5}	-	-	-	-	-	-
Rifampicin	10^{-3}	-	-	-	-	-	-
	10^{-5}	-	-	-	-	-	-
Ethambutol	10^{-3}	-	-	-	-	-	-
	10^{-5}	-	-	-	-	-	-
Positive control		-	9	+1	+1	+1	+1
Negative control		-	-	-	-	-	-

-: Absence, +: Presence

Table 3: International Union Against Tuberculosis and Lung Disease Scale

Reading	Calculation as
>500 colonies	4+
200-500 colonies	3+
100-200 colonies	2+
20-100 colonies	1+
1-19 colonies	Number of colonies recorded
No growth	Negative

+: Presence

CONCLUSION

Ethanolic extract of *A. keiskei* is a prospective natural anti-TB for the future.

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Conflicts of interest

There are no conflicts of interest.

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