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

**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 keskei* stem obtained from Lombok, Indonesia. The objectives of this study were to evaluate the sensitivity of *Mycobacterium tuberculosis* (Mt) H37Rv strain to *A. keskei* stem extract and to determine its minimum inhibitory concentration (MIC). The extraction methods of *A. keskei* 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. keskei* stem was assayed for their Mt inhibitory activity using the proportion method. The phytochemical analysis result showed that the secondary metabolites contain in the extract were flavonoid, polyphenol, tannin, monoterprenoid and sesquiterpen, quinon, and saponin. The anti-TB test result showed the active activity of ethanolic extract of *A. keskei* against Mt H37Rv strain with MIC ranging from 6% to 8% w/v. In conclusion, ethanolic extract of *A. keskei* is a prospective natural anti-TB for the future.

**Key words:** *Angelica keskei*, 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* (Mt), 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, Mt developed resistance against both the first-line and the second-line drugs.[3] Another study...
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.\textsuperscript{[1]}

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.\textsuperscript{[5,6]}

Of plant extracts, antimycobacterial compounds with the mechanism of activity have been reported.\textsuperscript{[7,8]}

\textit{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, \textit{A. keiskei} has been reported to show antimicrobial activity.\textsuperscript{[9]}
The chalcones were one of the compounds that had been exhibited the anti-TB activity.\textsuperscript{[10]}

Besides the activity, \textit{A. keiskei} also has been demonstrated \textit{in vivo} and the result is not toxic.\textsuperscript{[11]}

Therefore, the objectives of this study were to evaluate the sensitivity of Mtb H37Rv strain to \textit{A. keiskei} stems extract and to determine its minimum inhibitory concentration (MIC).

\textbf{MATERIALS AND METHODS}

\textbf{Plant materials}

The plant material used in this research is ashitaba stem (\textit{A. keiskei} [Miq.] Koidz.). The family name of this plant is \textit{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.

\textbf{Mycobacterium strain}

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

\textbf{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.

\textbf{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.\textsuperscript{[12]}

\textbf{Antituberculosis activity test}

Antibacterial activity of ashitaba stem extract against \textit{Mtb} H37rve 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.\textsuperscript{[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\textsuperscript{-3} and 10\textsuperscript{-5} in bacterial suspension 10\textsuperscript{-7}–10\textsuperscript{-9} 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.

\textbf{Mycobacterium sensitivity test on ashitaba extract}

The sensitivity test was done using Middlebrook 7H9 broth medium in BacT/ALERT 3D system.\textsuperscript{[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.

**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 (%w/v) | Bacterial suspension | Colony growth (weeks) |
|----------------------|----------------------|----------------------|----------------------|
|                      |                      | 10$^{-3}$            | 2       | 3       | 4       | 5       | 6       |
| Extract ashitaba     | 50                   |                     | -       | -       | -       | -       | -       |
|                      | 10                   |                     | -       | -       | -       | -       | -       |
|                      | 40                   |                     | -       | -       | -       | -       | -       |
|                      | 30                   |                     | -       | -       | -       | -       | -       |
|                      | 20                   |                     | -       | -       | -       | -       | -       |
|                      | 10                   |                     | -       | -       | -       | -       | -       |
| 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 antitubercular activity similar to the current study. 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 antimicrobial 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 dinucleotide plus hydrogen-cytochrome c reductase, and destroys cytoplasmic membranes achieving good combination effects against the rifampicin/streptomycin-resistant strain. 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.

CONCLUSION

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

Financial support and sponsorship Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. WHO. Global Tuberculosis Report. Geneva, Sweden: WHO Press; 2015.
2. Center of Data and Information of Ministry of Health of Republic of Indonesia. Tuberculosis: Temukan, Obati & Sembuh. Jakarta, Indonesia: Center of Data and Information Press; 2015. p. 2-7.
3. Singh MM. XDR-TB – Danger ahead. Indian J Tuberc 2007;54:1-2.
4. WHO. Global Tuberculosis Report. Geneva, Sweden: WHO Press; 2014. p. 13-5.
5. Tripathi RP, Tewari N, Dwivedi N, Tiwari VK. Fighting tuberculosis: An old disease with new challenges. Med Res Rev 2005;25:93-131.
6. Nayyar A, Jain R. Recent advances in new structural classes of anti-tuberculosis agents. Curr Med Chem 2005;12:1873-86.
7. García A, Bocanegra-García V, Palma-Nicolás JP, Rivera G. Recent advances in antitubercular natural products. Eur J Med Chem 2012;49:1-23.
8. Copp BR, Pearce AN. Natural product growth inhibitors of *Mycobacterium tuberculosis*. Nat Prod Rep 2007;24:278-97.
9. Caesar LK, Cech NB. A review of the medicinal uses and pharmacology of ashitaba. Planta Med 2016;82:1236-45.
10. Lin YM, Zhou Y, Flavin MT, Zhou LM, Nie W, Chen FC, *et al.* Chalcones and flavonoids as anti-tuberculosis agents. Bioorg Med Chem 2002;10:2795-802.
11. Maronpot RR. Toxicological assessment of ashitaba chalcone. Food Chem Toxicol 2015;77:111-9.
12. Fansworth NR. Biology and phytochemical screening of plants. J Pharm Sci 1966;55:263-4.
13. Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. Bull World Health Organ 1969;41:21-43.
14. Nayak N, Bajpai M, Razdan B. Plumbagin analogs-synthesis, characterization, and antitubercular activity. J Adv Pharm Technol Res 2014;5:29-32.
15. Sembiring B, Manoi F. Identifikasi mutu tanaman ashitaba. Bull Littro 2011;22:177-85.
16. Kemenkes RI. Pedoman Nasional Pengendalian Tuberkulosis. Jakarta: Kementrian Kesehatan RI; 2012.
17. Copp BR. Antimycobacterial natural products. Nat Prod Rep 2003;20:535-57.
18. Gautam R, Saklani A, Jachak SM. Indian medicinal plants as a source of antimycobacterial agents. J Ethnopharmacol 2007;110:200-34.
19. Askun T. The significance of flavonoids as a potential anti-tuberculosis compounds. RRJPTS 2015;3:1-12.
20. Grange JM, Snell NJ. Activity of bromhexine and ambroxol, semi-synthetic derivatives of vasicine from the Indian shrub Adhatoda vasica, against Mycobacterium tuberculosis in vitro. J Ethnopharmacol 1996;50:49-53.
21. Navarro-García VM, Luna-Herrera J, Rojas-Bribiesca MG, Álvarez-Fitz P, Rios MY. Antibacterial activity of Aristolochia brevipes against multidrug-resistant Mycobacterium tuberculosis. Molecules 2011;16:7357-64.
22. Taylor RS, Edel F, Manandhar NP, Towers GH. Antimicrobial activities of Southern Nepalese medicinal plants. J Ethnopharmacol 1996;50:97-102.
23. Singh R, Hussain S, Verma R, Sharma P. Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of Cassia sophera and Urtica dioica. Asian Pac J Trop Dis 2013;6:366-71.
24. Dubey D, Rath S, Sahu MC, Debata NK, Padhy RN. Antimicrobials of plant origin against TB and other infection and economics of plant drugs-instropection. Indian J Tradit Knowl 2012;11:225-33.
25. Fauziyah PN, Sukandar EY, Ayuningtyas DK. Combination effect of antituberculosis drugs and ethanolic extract of selected medicinal plants against multi-drug resistant Mycobacterium tuberculosis isolates. Sci Pharm 2017;85. pii: E14.