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Full Length Research Paper

Petiveria alliacea: New alternative for the treatment of sensitive and multi-resistant *Mycobacterium tuberculosis*

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The purpose of this study was to determine the activity of *Petiviera alliacea* extracts against drug-sensitive and resistant strains of *Mycobacterium tuberculosis. P. alliacea* was extracted with ethanol using the maceration method. The proportional method was used to test the anti-mycobacterial activity in ethanol extract against drug-sensitive and resistant strains of *M. tuberculosis.* Drug-sensitive and resistant H37Rv strains of *M. tuberculosis* were resistant to ethambutol and isoniazide (EH) and resistant to streptomycin and rifampicin (SR). Tests were conducted *in vitro* on Lowenstein Jensen (LJ) medium that was mixed with various concentrations of extracts, inoculated with bacterial suspensions, and incubated at 37°C. Microbial growth was observed every week starting from Week 4 to Week 8 after inoculation to rule out possibility of delayed suppressed growth. Standard drugs were used for comparison. The ethanol extract of *P. alliacea* was found to be active against *M. tuberculosis* at concentrations of 1280 and 2560 µg/ml. it was concluded that the activity of *P. alliacea* inhibits the growth of *M. tuberculosis* better than standard drugs. Further testing is needed to identify its beneficial components.

Key words: *Petiveria alliacea*, anti-mycobacterial, *Mycobacterium tuberculosis*.

INTRODUCTION

Infectious disease is still the highest contributor to morbidity and mortality in developing countries, including Indonesia. *Mycobacterium tuberculosis* has infected one-third of the world's population. It is estimated that 9 million people worldwide were infected with tuberculosis (TB), and 2 million people die from tuberculosis every year. Ninety-five percent of patients with tuberculosis were in developing countries (Karakousis et al., 2004; Dye et al., 1999; WHO, 2004; 2006; 2009; Centres for Disease Control, 2005). Tuberculosis in humans can damage body tissue, and symptoms include inflammation of the lining of the lungs and vague pain, cough, fever and weight loss. Tuberculosis is typically treated with isonicotinic acid hydrazide (INH) or streptomycin and para-aminosalisilate, given in combination. *M. tuberculosis* commonly become resistant to standard tuberculosis drugs. This resistance occurs as a result of monotherapy or therapy that is not given for enough time. At least two kinds of anti-tuberculosis drugs should be given for effective therapy (Global Tuberculosis Control, 2009; James, 2006). These bacteria become resistant easily to one or several types of anti-tuberculosis standard drugs (multidrug resistant, MDR), or to two to four types of standard anti-tuberculosis drugs (extensively drug-resistant tuberculosis [XDR-TB]); so often the treatment is not successful (Centres for Disease Control, 2005). Based on resistance data, it is necessary to develop new drugs to control tuberculosis, particularly against MDR and XDR. There is also a need to alleviate

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the shortage of current drug regimens by developing a safer, more effective and more affordable drug with a shorter treatment time (James, 2006).

Petiveria alliacea belongs to the Phytolaceae family, known as singawalang in traditional uses in Indonesia. It grows in the Caribbean, Latin America, West Africa and other regions. For hundreds of years it has been used for pain relief, and as an anti-influenza, anti-inflammatory, anti-tumor, anti-bacterial, anti-fungal, anti-hyperlipidemia, and anti-diabetic drug (Tropical Plant Database-Anam, 2011). This plant also grows in Indonesia, but it has not been used extensively. It is traditionally used in Indonesia as an analgesic, anti-inflammatory and for treatment of hemoptysis. Ethnobotany tests were conducted in Bogor, and they showed that the use of *P. alliacea* can reduce the length of therapy with standard drugs in tuberculosis patients (Widowati, 2007; Weniger et al., 1986).

Pharmacological studies have tested the efficacy of *P. alliacea* and the results have been published in several international journals, demonstrating its use as anti-cancer, imunomudolator and anti-infective, which has been documented in PubMed (Kubec et al, 2003). Research activity against drug-resistant *M. tuberculosis* is still very minimal.

This study tested the activity of *P. alliacea* leaf against drug-sensitive and resistant *M. tuberculosis* strain H37Rv, resistant to ethambutol and isoniazide (EH) and resistant to streptomycin and rifampicin (SR). The *M. tuberculosis* H37Rv strain is defined to be sensitive, because bacterial growth could be inhibited by INH rifampicin, EH is resistant against ethambutol and isoniazid, and SR is resistant against streptomycin and rifampicin (Weyer, 1997; Tuberculosis Cluster Bureau of AIDS, TB, and SITs, 2007).

The purpose of this study was to determine the activity of *P. alliacea* extracts against drug-sensitive and resistant *M. tuberculosis*.

MATERIALS AND METHODS

Plants

Singawalang plants were obtained from the Cilendek area in Bogor based on an ethnobotany survey in one village in Bogor. The leaves were picked from the first level down to five levels after major upper leaves from a Singawalang crop with a height of 80 to 120 cm. Plant identification was conducted by the Department of Herbarium Bandungense, School of Biological Science and Technology, Bandung Institute of Technology.

Processing includes sorting, washing, chopping, drying, and grinding of the raw leaves into powder simplicia. 200 mg of dry leaf powder was extracted with 1 L of 96% ethanol using maceration for 24 h and were filtered. Filtration was done with Whatmann 42 filter paper (125 mm). Then, the residue was macerated twice with 9% ethanol. The filtrate obtained was concentrated with a vacuum rotary evaporator (Buchi, Switzerland), at 50 to 60°C. The extract was concentrated again in a water bath at 50 to 60°C to obtain a viscous extract. The extract was stored in a refrigerator maintained at 5 to 10°C.

Bacteria

The *M. tuberculosis* used in this study consisted of three strains-strain H37Rv sensitive, EH (resistant to ethambutol and INH) and SR (resistant to streptomycin and rifampicin). Mycobacteria were obtained from the Institute for Health Development Laboratory, Bandung, which is licensed by the World Health Organization as one of the laboratories that meets the eligibility in tuberculosis drug-susceptibility testing. Cultures of *M. tuberculosis* (H37Rv), EH and SR strains were grown on LJ medium and maintained at -70°C. The culture revived from −70°C were sub cultured on Middlebrook 7H9 broth.

Media

LJ media (Merck) of 18.75 g was dissolved in 300 ml of sterile aquabidest, then added to 5 ml of glycerol phosphatidic acid. The media was mixed until it was homogeneous and then was sterilized in an autoclave at 121°C for 20 min. Once cool, 500 ml of duck egg yolk was added and stirred again until homogeneous.

Anti-mycobacterial testing by the proportional method

Condensed extract was dissolved in dimethyl sulfoxide (DMSO, Sigma), then stored as the main solution at 5 to 10°C until used. Extracts were tested at concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 µg/ml against the LJ medium. The standard drugs rifampicin (Merck) 40.0 µg/ml, isoniazid (Merck) 0.2 µg/ml, ethambutol (Merck) 2.0 µg/ml, and streptomycin (Merck) 4.0 µg/ml were used as drugs comparator. DMSO solution of 5% v/v was used as a control group. Stock solution of the crude extracts was prepared using 1% DMSO. It was verified that DMSO did not suppress or delay the growth of *M. tuberculosis* strains (Grange and Snell, 1996).

Then, the solution of extracts, comparator drugs and DMSO were added to the tubes containing LJ media in order to obtain a total volume of 5.0 ml under desired concentration and was stirred until homogeneous. Appropriate volumes of compounds were incorporated into duplicate plates of LJ media. The tube was tilted and put into an oven at 85°C for 1 h for the medium to become solid.

Inoculation was performed on drug-sensitive and resistant strains H37Rv *M. tuberculosis*. Each bacteria was suspended in 0.9% NaCl to obtain a turbidity of McFarland (MCF) 10^{-3} and 10^{-5}. Each 0.1 ml of bacterial suspension was inoculated on the surface of the media containing extracts, comparator drug and solvent. All tubes were incubated (WTB Binder) at 37°C. The growth of bacterial colonies was observed weekly starting from week 4 to week 8 (Tuberculosis Cluster Bureau of AIDS, TB, and SITs, 2007; WHO-CD-TB, 2001).

Evaluation of anti-mycobacterial activity

Anti-microbacterial activity tests were performed *in vitro* against drug-sensitive and resistant strains H37Rv *M. tuberculosis*. Strains H37Rv sensitive can be killed by the standard anti-tuberculosis drugs (ATD). EH strains were already resistant to isoniazid and ethambutol, while SR strains were resistant to streptomycin and rifampicin.

Anti-mycobacterial activity testing was done using the proportional method. In this method, we used two concentrations of

bacterial inoculum of McFarland 10^{-5} and 10^{-3} containing approximately 10^2 and 10^4 colony/ml. It aimed to obtain the number of colonies (50 to 300) which can be calculated at one concentration to determine the total number of colonies. The number of bacteria colonies were measured to determine the activity of ATD; extracts were compared to the total bacterial population of the control group. The number of *M. tuberculosis* colonies demonstrated the activity of the extracts and ATD. The sensitivity of *M. tuberculosis* against extracts and drug sensitivity test (DST) *in vitro* was demonstrated by the lower or equal number of colonies as compared to the positive group comparison drug. This method can be used to obtain ATD resistance data indicated by comparing the number of mutant colonies to the total population of the bacteria tested control group. Bacteria is said to be resistant against an antibacterial agent when the percentage of the ratio is ≥ 1% (Tuberculosis Cluster Bureau of AIDS, TB, and SITs, 2007; WHO-CD-TB, 2001).

Phytochemical screening

The examination of the chemical compounds of ethanol extract of *P. alliacea* was conducted to determine the class of the main compounds present in the extract that was responsible for anti-tuberculosis activity. Standard procedures described by the Indonesian Department of Health were used to screen alkaloid, tannins, saponins, flavonoids and terpenoids compounds (Departemen Kesehatan RI, 2000).

RESULTS

Table 1 shows the activity against *M. tuberculosis* strain H37Rv, EH and SR at various concentrations of ethanol extracts of *P. alliacea* and various comparator drugs. Ethanol extract has anti-mycobacterial activity at a concentration of 1280 µg/ml against all strains of *M. tuberculosis* where there is no growth of colonies of *M. tuberculosis*. The ethanol extract of *P. alliacea* leaves has a better activity in *M. tuberculosis* resistant strain than comparator drugs.

Figure 1 shows the percentage of colonies of *M. tuberculosis* that were exposed by the extracts. The percentage of colonies decreased in extracts with various concentrations and decreased number of colonies for *M. tuberculosis*. The results of phytochemical screening showed that the ethanol extract of *P. alliace* contains alkaloid, flavonoids and tannins compounds. Anti-mycobacterial activity against drug-sensitive and resistant strains H37Rv of *M. tuberculosis* from *P. alliace* is reported for the first time.

DISCUSSION

The *in vitro* testing of anti-mycobacterial drugs was done with the proportional method; this method's accuracy has been recognized by WHO (WHO-CD-TB, 2001; APHL, 2007). Growth of strains H37Rv of *M. tuberculosis* was detected on the normal LJ medium control bottle after 4 weeks and by the 8 weeks, there was good growth. DMSO control also showed similar growth by 8 weeks, while growth was completely inhibited in the "test" bottle inoculated with *M. tuberculosis* pre incubated with *P. alliacea* extracts for 8 weeks at 37°C.

The ethanol extract of *P. alliacea* leaves have anti-mycobacterial activity against drug-sensitive and resistant strains H37Rv of *M. tuberculosis,* namely bacteria-sensitive H37RV, bacteria-resistant EH (resistant to ethambutol and isoniazid) and SR (resistant to streptomycin and rifampicin). The minimum inhibitory concentration (MIC) for all types of *M. tuberculosis* is 1280 µg/ml, which is shown by the absence of bacterial growth, and nearly all concentrations showed a decreased number of bacterial colonies as compared to controls (Table 1). DMSO as the solvent extract is often used in testing antimycobacteria, as well as the DMSO is added to LJ medium for the control group.

Percentage growth in the number of colonies of *M. tuberculosis* against all the three strains were observed for both concentrations of 1280 and 2560 µg/ml ethanol extract; *P. alliacea* is 0% (Figure 1). When compared with the control group, almost all of the concentration indicates the reduction of percentage in colony growth. All "test" bottles were further incubated up to 8 weeks to rule out the possibility of delayed suppressed growth. Growth of *M. tuberculosis* was completely inhibited during the incubation of 4 to 8 weeks, indicating static or incomplete killing by the phytochemical contained in the *P. alliacea* extract. *P. alliacea* extract showed an advantage over comparable medicines, because it can be very active against strains resistant to rifampicin, isoniazid, streptomycin and ethambutol. Previous testing on *P. alliacea* showed a good anti-bacterial and anti-fungal activities. A collection of thiosulfinates and sulfines compounds contained in *P. alliacea* is suspected to be the major component responsible for anti-bacterial and anti-fungal activities (Kim et al., 2006; Benevides et al., 2001; Kubec et al., 2003). Secondary metabolites from natural products and some of their derivatives have been reported to exhibit remarkable growth inhibitory activity towards *M. tuberculosis* and some of them have been selected as prototype molecules for the development of new antitubercular agents (Triphati et al., 2005; Nayyar et al., 2005). These secondary metabolites isolated from plants, bacteria, fungi, marine organisms and algae, were grouped according to their chemical type as terpenes (sesquiterpenes, diterpenes, sesterterpenes, triterpenes), steroids (sterols), alkaloids (indole, quinoline, pyridoacridone, and manzamine alkaloids, etc), aromatics(flavonoids, chalcones, coumarins, lignans, xanthones, anthracenes, anthraquinones, naphthalenes, chromones, etc), polyketides (acetylenic fatty acids, polycyclic esters, quinones, etc), and peptides (Gracia et al, 2012). Some of the compounds are positive in our first screening. However, this study is the first to report on the

Table 1. Anti-tuberculosis activity against drug-sensitive strain H37Rv and resistant EH and SR *M. tuberculosis* at various concentrations of ethanol extract of *P. alliacea*, using DMSO as a control group and various comparator drugs.

Concentration (µg/ml of medium)	Number of colony		
	H37Rv sensitive	Resistant H37Rv EH	Resistant H37RvSR
5	145	99	138
10	93	77	110
20	98	87	120
40	90	73	115
80	67	73	114
160	53	72	88
320	58	71	77
640	61	71	88
1280	No growth	No growth	No growth
2560	No growth	No growth	No growth
DMSO (control)	176	195	167
Isoniazid (0.2)	No growth	175	No growth
Rifampisin (40)	No growth	No growth	122
Ethambutol (2.0)	No growth	45	No growth
Streptomycin(4.0)	No growth	No growth	85

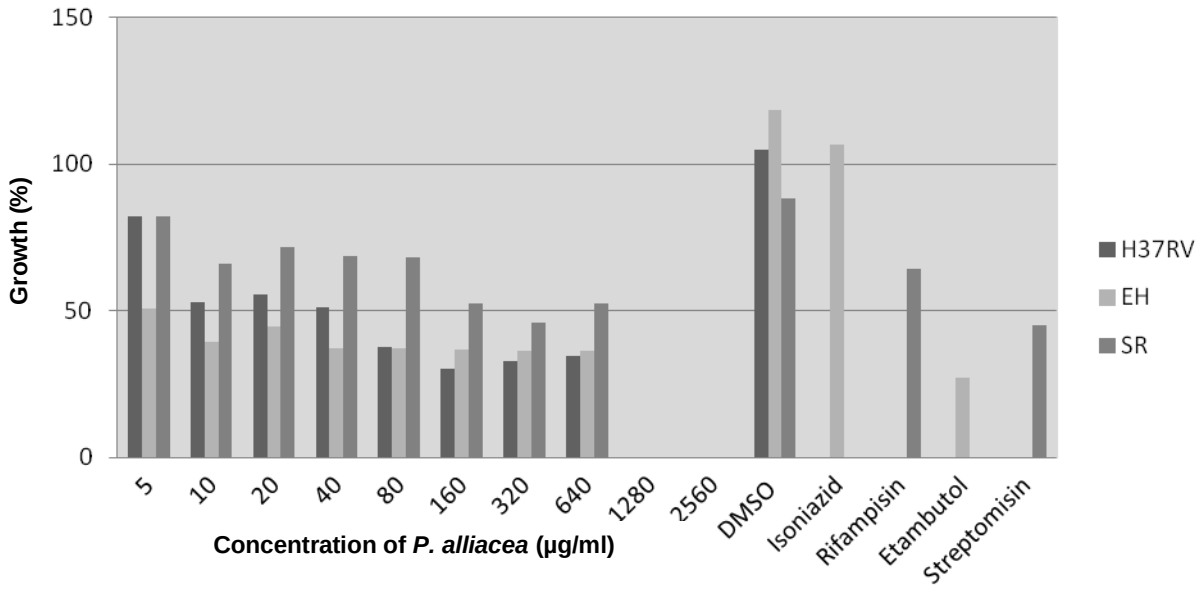

Figure 1. Growth percentage of the number of *M. tuberculosis* colonies of strains H37RV, EH and SR at various concentrations of ethanol extract of *P. alliacea*, using DMSO as a control group and comparator drugs against control.

efficacy of anti-mycobacterial activity of *P. alliacea* against resistant and sensitive bacteria. This discovery comes at a good time, because of the rising cases of MDR. Activity of the extract was relatively lower as compared to the standard drugs, this is due to the test using a crude extract, which is undergoing the process of fractionation. Isolation of compounds is in progress, which is expected to deliver a compound or group of compounds responsible for the anti- mycobacterial activity. This can be improved if the main compounds responsible for anti-bacterial activity could be found and isolated from the extract.

It is necessary to investigate parts of the plants that showed better anti-mycobacterial activity and comprehensive

phytochemical analysis of plant extracts. Further detailed phytochemical screening and bio activity studies need be carried out using crude solvent extracts and purified constituents to comprehend their role in anti-mycobacterial activity. This study opens the possibility of obtaining novel compounds for the treatment of TB, including MDR treatment. Isolation of active compounds, pharmacology and toxicology studies are currently underway and will be reported later.

Conclusion

The ethanol extract of *P. alliacea* could be useful in the development of new antimicrobial drugs, especially against infections caused by three strains of *M. tuberculosis*. This study also supports the use of plants to treat infections associated with the microorganisms studied so that the cultivation of these plants in Indonesia could be implemented with promising results.

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