



UTILIZATION OF NEMBA STEM AS AN ANTITUBERCULOSIS COMPARED WITH RIFAMPICIN AND ETAMBUTOL

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ABSTRACT

Indonesia ranks third in the number of *tuberculosis*, after India and China. Treatment of *tuberculosis* takes a long time, and the lack of discipline in taking medication has caused the *Mycobacterium tuberculosis* to be resistant to many synthetic drugs that have been used so far. In contrast, the discovery of new synthetic drugs is very slow. Traditionally, neem bark (*Azadirachta indica* JUSS.) has been used to treat coughing up blood and phlegm, and the results of previous studies show that ethanol extract of neem bark *in vitro* can inhibit the growth of *Mycobacterium tuberculosis*. This study was conducted to determine the toxicity of neem bark extract, formulation of neem bark ethanol extract into tablet preparations, and the potential of this tablet preparation as anti-tuberculosis *in vivo* in animals. Neem bark extract was made by percolation using an ethanol extractor. Toxicity test was carried out in the form of an acute toxicity test on mice to calculate the LD50 value and histopathological observations of the liver, lungs, and intestines. The tablets were made by wet granulation. The potential test of tablet preparations as anti-tuberculosis was performed *in vivo* on animals infected with *Mycobacterium tuberculosis* H37Rv using a nebulizer. The results showed LD 50 = 11.85 ± 0.571 , including mild toxic. The ethanolic extract of neem bark can be formulated into tablets using a 6% gelatin binder, Manihot starch and **primojel** as a binder, and a mixture of starch and lactose as a filler. Neem bark extract tablets can cure tuberculosis of experimental animals that have been infected with *Mycobacterium tuberculosis* H37RV, which is given a dose of 3 times a day two tablets (50 mg/tablet) for six weeks seen tuberculosis bacteria from +3 to negative, and a dose of 3 times a day once a day. Tablet looks from +3 to +1.

Keywords: Ethanol extract of neem bark, Toxicity test, Tablet manufacture, Anti-tuberculosis, effectiveness test in guinea pigs.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, which infects latent or progressively transmitted from person to person through coughing. Being in close contact through breathing with TB sufferers will increase the possibility of transmission. *Mycobacterium tuberculosis* is an acid-fast bacterium that is different from other bacteria because it grows very slowly and resistance develops very quickly. The slow dividing nature of this bacterium is one of the factors that discovers anti-tuberculosis drugs much more complex and slower than other antibacterials (Ganiswara, 1995). Tuberculosis is a threat to the Indonesian

population. In 2004, as many as 250,000 new patients were added, and about 140,000 deaths occurred every year. Most people with tuberculosis are productive age between 15-55 years. This disease is the third leading cause of death after heart disease and acute respiratory disease in all ages (Depkes RI, 2005).

The increase in the number of tuberculosis sufferers is caused by various factors, one of which is the lack of patient adherence to taking medication, because the treatment of this disease takes a long time, which is approximately six months, expensive drug prices, the emergence of multiple resistances, lack of host resistance to mycobacteria, reduced bactericidal power of

existing drugs and the economic crisis (Ministry of Health RI, 2005). Drugs that have been commonly used in the treatment of tuberculosis infection are isoniazid, pyrazinamide, rifampin, ethambutol, and streptomycin. Most people with TB can be treated with these drugs. Provide optimal therapy. However, due to the possibility of microbial resistance or certain factors from the patient that limits the use of the drug, treatment with other drugs is needed (Wattimena, JR et al., 1991)

Traditionally, the neem plant (*Azadirachta indica* Juss) is used to cure various diseases, including Stew bark used to treat coughing up phlegm and bleeding. (Dalimarta, 1999).

In a previous study (Ambarwati, 2007), neem seeds could inhibit the growth of *Salmonella thyposa* and *Staphylococcus aureus*. Inhibit the growth of *Streptococcus mutants* and *Streptococcus faecalis* (Almas, 1999), neem oil to inhibit *E. coli* and *Klebsiella pneumoniae* (Sai Ram et al., 2000), neem as a mouthwash to inhibit *Streptococcus mutans* and *Lactobacillus* (Vanka et al., 2001), and the ethanolic extract of neem bark can inhibit the growth of *Mycobacterium tuberculosis* in vitro, (Fatimah, 2012),

Referring to the efficacy of neem bark which has been used traditionally as a cough medicine for bloody coughs even for chronic bloody coughs, and the results of previous research extracts neem bark can inhibit the growth of tuberculosis bacteria, so it is very likely that neem bark can be developed into alternative anti-tuberculosis drugs and natural ingredients.

In this study, the researchers tested the toxicity of the ethanol extract of neem bark on mice, the formulation of the neem bark ethanol extract into tablet preparations, and tested the anti-tuberculosis activity of these tablets in vivo in guinea pigs induced with *Mycobacterium-tuberculosis*. will be able to obtain an overview of the toxicity of the bark of neem and its effectiveness as anti-tuberculosis in animals, so it is hoped that the bark of neem can be developed into an alternative medicine from materials for the treatment of tuberculosis.

MATERIALS AND METHODS

Plant material is neem bark that has aged brown. Chemical media: OGAWA media, potassium dihydrogen phosphate, magnesium

citrate, sodium glutamate, glycerin, malachite green, methylene blue, fuchsin, toluene, rifampin, ethambutol, aqua dest and the bacterium *Mycobacterium tuberculosis* H37Rv (from the Medan Regional Health Laboratory). The tools used are glassware, water content determination tools, tools for toxicity tests, tools for tablet printing and physical test of tablets, microbiological tools to test the effectiveness of tablets as anti-tuberculosis in experimental animals and nebulizers, experimental animals, mice, and guinea pigs

. Stages of work:

- Collection of neem bark, dried and powdered into simplicia powder, phytochemical screening, and simplicia quality test.
- Making extract by percolation method with 96% ethanol solvent, filter
- Making reagents and Ogawa media for culture examination and testing.
- Identification of tuberculosis bacteria by Ziehl- Neelsen, and cultivation on OGAWA media
- Acute toxicological test of ethanolic extract of neem bark in mice
- Preparation of tablets from ethanolic extract of neem bark
- Test the anti-tuberculosis of neem bark tablets in guinea pigs compared to rifampin and ethambutol.

Toxicological

- test The test was carried out using extract suspended at 50% concentration using CMC
- . The experimental animals used were 30 healthy male mice, aged 2 — 3 months with a bodyweight of 20 — 30 grams, acclimatized for seven days, then the animals were divided into five groups. Treatment (WHO, 1993).
 - Control group (K), was given CMC suspension solution
 - . Treatment group I(P1) was given test solution dose I
 - , treatment group II(P2) was given test solution, dose II
 - , treatment group III(P3) was given test solution, dose III
 - , treatment group IV(P4) was given Test solution IV dose
 - of treatment group V(P5) Given test

solution dose V

Toxicity test is carried out with the working stages of determining the dose carried out in 3 stages, namely the first stage (dose orientation), the second stage test (preliminary test), and the third stage test (actual test), calculated LD50 using the Thompson Weil method and histopathological tests, to determine the LD50 value, a graded dose consisting of five doses was used. Physical observations of toxic symptoms were carried out for 24 hours intensively on all groups of mice. Then observations were continued for 14 days and then counted the number of mice that died from each group. Then the LD50 is determined from the data using the Thompson Weil formula:

$$\text{Range LD}_{50} = \text{antilog} (\text{Log } m \pm 2 \times \log m)$$

Information:

- m = LD₅₀
D = Smallest dose given
d = Log Multiple Dose
f = Factor (Weil table Calculations)

The histopathological picture was taken from the liver and kidneys. The organs were then washed in distilled water and then put into a pot containing 10.% formalin buffer for further preparation of preparations to see the histopathological picture.

Making tablets

Tablets were made by wet granulation, formulated using neem bark extract 50 mg each tablet, and the weight of each tablet is 300 mg, with various variations of adjuvants:

1. Variation of binder: gelatin (4% gelatin, 6% gelatin, 8% gelatin, starch (mucilago amili 10). %, mucilago amili 12.5%, and , mucilago amili 15% (F3), and Avicel (Avicel 2%, Avicel 3%, Avicel 4%.
2. Variation of developer material: starch Manihot 5%, CMC 5%, and primojel 5%
3. Variation of fillers: starch, Avicel, lactose, and a mixture of starch and lactose.

The resulting tablets were evaluated with the parameters of friability, hardness, weight uniformity, and disintegration time.

in vivo

an anti-tuberculosis test of neem bark extract tablets was carried out on guinea pigs induced with *Mycobacterium tuberculosis* H37RV

by spraying into the bronchi using a nebulizer as much as 3 ml at intervals of 24 hours for seven days. Consecutively, it continued every two days for seven days and every three days for up to 7 days. Furthermore, 50 ml of spray was taken from the esophagus to test *Mycobacterium tuberculosis*, which has been positive for tuberculosis. Furthermore, the animals were given test material according to each group 3 times a day, and specimens were taken every week 4-5 times.

Specimens were taken from animals induced in the form of a bronchial spray, put into a sterile container, followed by identification and cultivation of *Mycobacterium tuberculosis* by the specimen was transferred to a test tube, added with phosphate buffer pH 7, homogenized until wholly suspended. Then 0.1 ml pipette was pipetted and inoculated into two tubes containing Ogawa media, flattened over the entire surface of the media, incubated at 37°C for 6 weeks and observed for growth every week with the reading criteria: (Japan International Cooperation Agency, 1987)

(-): no growth

(+1): visible few yellow colonies 1-200 colonies

(+2): ^{1/2}by yellow colonies (200-500 colonies)

(+3): ^{3/4}of the media covered by yellow colonies (500-2000 colonies)

(+4): media wholly covered by yellow colonies (more than 2000 colonies (Japan International Cooperation Agency, 1987)

RESULTS AND DISCUSSION

Toxicological Test Results :

LD50

The first stage of the test was carried out as a test orientation to determine the dose to be given in the next test, given the test material with an increase in the dose of a multiple of two, and observed for 24 hours until a dead animal is obtained. The results of the first stage test (dose orientation) are seen as 20% mortality in a group (P4) at a dose of 80 mg/20 g BW. Based on these results, it can be continued to the second stage of the test (preliminary test) using the smallest dose taken from the results of the first stage of the test, namely the dose that is close to the dose at the death of 20%. In the second stage of the test, the smallest dose close to the dose of 80 mg/20 g BW is used, that is, 50 mg/20 g BW is used because at 40 mg/20 g BW, no animal deaths have been seen. Furthermore, the dose was increased by a

multiple of two, and observations were made for 24 hours. The results of the second stage of the test (preliminary test) showed 0% mortality in groups P1) and (P2). Based on these results, it can be continued to the third stage of the test (actual test) using the initial dose determined based on the highest dose causing 0% mortality in the second stage of the test (preliminary test) which is 100 mg/20 g BW. In the third stage of the test, the smallest dose of 100 mg/20 g BW is given, then the dose is increased by multiples of the dose based on the results of the calculation of the price R = 1.4142:

$$R = \text{antilog}$$

$$d = \frac{\log \log \text{ multiples of dose}}{\text{number of group} - 1}$$

The multiple of dose between group and group 4 is 4 then:

$$d = \frac{\log \log 4}{4}$$

$$d = \frac{0.602}{4} = 0.15051$$

$$R = \text{antilog } 0.15051 = 1.4142$$

and observed for 14 days. The results of the third stage of the test (actual test), a single dose in male mice can cause death at several dose levels. Then, based on this data, the LD50 value was calculated using the Thompson Weil formula, obtained an LD50 of 11.85 g/kg BW orally in male mice (*Mus musculus* Linn). The calculation is as follows:

$$\text{Log } m = \text{Log } D + d (f + 1)$$

$$D = 141.42$$

$$d = 0.15051$$

$$f = \text{in the Weil table } r = 1,3,3,2 \text{ is } 0.50000$$

$$\text{Log } m = \text{Log } D + d (f + 1)$$

$$\text{Log } m = \text{Log } 141.42 + 0.15051 (0.50000 + 1)$$

$$\text{Log } m = 2.1505 + 0.15051 (1.500000)$$

$$\text{Log } m = 2.1505 + 0.2252$$

$$\text{Log } m = 2.3757 \text{ mg/20 g BW}$$

$$m = \text{antilog } 2.3757 = 237.54 \text{ mg/ } 20 \text{ g BW}$$

$$\text{LD } 50 = 0.237 \text{ g/20 g BW}$$

$$\text{LD } 50 = 11.85 \text{ g/kg BW}$$

Then the LD50 range is calculated and the LD50 range is (11.85 ± 0.571) mg/kg BW.

$$\text{Range LD50} =$$

$$\text{antilog} (\log m \pm 2 \times \log m$$

$$f = \text{in the Weil table } r = 1,3,3,2 = 1.90394$$

$$d = 0.150151$$

$$m = \text{LD50 value} = 0.237 \text{ g/20 g BB}$$

$$\log m = 0.150151 \times 1.90394$$

$$\log m = 0.28587$$

$$\text{Range LD50} =$$

$$\text{Antilog} (\log m \pm 2 \times \log m)$$

$$= 0.237 \pm 2 \times 0.28587$$

$$= (0.237 \pm 0.57175) \text{g/20 g BW}$$

$$= (11.85 \pm 0.57) \text{g/kg BW}$$

Based on the LD50 range, toxicity criteria were determined using the criteria of Frank C. Lu, 1995

1. Super toxic
5 mg/kgBW or less
2. Very Very Toxic
5 - 50 mg/kgBW
3. Very Toxic
50 - 500 mg/kgBW
4. Moderate Toxic
5g/kgBW
5. Mild Toxic
– 15 g/kgBW
6. Practically Non-Toxic
> 15 g/kgBW

The calculation results of the LD50 value range and adjusted for the toxicity criteria indicate that the ethanolic extract of neem bark (*Azadirachta Cortex*) can be classified into the "Mild Toxic" criteria. " single dose of neem bark ethanol, summarized in table 5 below, died.

Histopathological Examination Results The

the interpretation of histopathological preparations in test animals showed damage to the liver and kidney in each treatment group and control group. Histopathological examination of the liver and kidneys can be seen based on observations of several random fields of view, the degree of damage to hepatocytes in the liver of the test animal group (P2) is greater than the group (P1), group (P3) is more significant than (P2), group (P2) P4) is more significant than (P3). The group (P5) is more significant than (P4). This means that increasing the dose of neem bark ethanol extract can increase the toxic effect on the liver of the test animals. Histopathological observations of the kidneys showed necrosis based on the constriction that occurred in the proximal tubule around the glomerulus. In the control group, the smallest percentage was 5%, and so the percentage increased following the dose stages from the lowest to the highest. Moreover, the highest percentage of kidney

necrosis was in the group (P5) with a dose of 400 mg/20g BW with 20%. The control group also showed the picture of necrosis but with a small percentage. The degree of damage to the proximal tubular epithelial cells in the kidneys of the test animals (P2) was more significant than the group (P1), the group (P3) was more significant than (P2), the group (P4) is more significant than (P3), and the group (P5) is more significant than (P4). This means that increasing the dose of neem bark ethanol extract can increase the toxic effect on the kidney organs of the test animals.

These results prove that administering a single dose of neem bark ethanol extract with an observation period of 14 days resulted in liver and kidney damage in male mice (*Mus musculus* Linn). Moreover, there is a difference in the percentage of degrees of damage in the histopathological picture of the liver and kidneys of the test animals, from the smallest percentage in the control group and the highest percentage in the highest dose group on the administration of ethanol extract of neem bark. They are dosed orally. And it is suspected that liver cell damage is caused by the active compound of neem bark. Biswas et al. (2002) explained that neem plants contain active compounds such as azadirachtin, salanine, meliantriol, nimbin, nimbolide, gedunin. Nimbolide has a toxic effect on the body; this statement follows the research of Glinkuson (2002), which explains that giving nimbolide at a dose of 225 mg/kg BW in mice can cause necrosis in the liver, necrosis in the kidneys, and hemorrhage in the intestines. However, this toxic effect is seen in very large doses.

Results of tablet

Tablet formulation with a dose of 50 mg per tablet was made with a diameter of 11 mm using a variety of auxiliary materials. It can be seen that the tablet is good from the evaluation of several parameters, namely:

- a. The best binder is 6% gelatin because tablets that use other binders do not meet the requirements. In terms of friability and hardness of tablets, the resulting tablet is brittle.
- b. The developer of starch manihot or primojel gives good results, while the CMC is not good because the resulting tablets show a long disintegration time.
- c. filler good is in the form of a mixture of

starch and lactose

In vivo Anti Tuberculosis Test Results in experimental animals

Anti TB test on animals was carried out in 5 groups: blank, control was given Rifampicin, control was given ethambutol, and 2 test groups each given three times a day one tablet, and three times a day two tablets, consecutively for four weeks, then continued administration accompanied by every seven days taken 50 ml of spray water in the bronchi to observe the reduction of bacteria implanted in OGAWA media for 6-8 days week. The results showed inhibition of bacterial growth at four times of taking.

Result: The number of bacteria before administration of the test material/drug was +3

group: changed from +3 to +4 on the first to fourth intake.

Control group: changed from +3 to negative TB on the third take

Group dose three times a day two tablets: changed from +3 to negative on the fourth intake. Dosage group three times daily one tablet: changed from +3 to +1 on the fourth intake.

CONCLUSION

The neem bark extract obtained LD 50 = (11.85 ± 0.571) , was at range (5 - 15) g/kg BW; it is included in the mild toxic category. The ethanolic extract of neem bark can be formulated into a tablet dosage form of 50 mg per tablet with a weight of 300 mg per tablet using suitable adjuvants, namely: 6% gelatin binder, starch developer Manihot and primojel, and an equal amount of starch and lactose mixture filler. Neem bark extract tablets can cure tuberculosis in experimental animals that have been induced with *Mycobacterium tuberculosis* H37RV, which is given a dose of 3 times a day two tablets (50 mg/tablet) for six weeks seen tuberculosis bacteria from +3 to negative, while at a dose of 3 times a day one tablet looks from +3 to +1.

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