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The Efficacy of Soursop Leaf Extract as a Hepatoprotector in White Rats Rattus norvegicus Induced by the Antituberculosis Drugs Isoniazid and Pyrazinamide

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ABSTRACT

The use of antituberculosis drugs isoniazid and pyrazinamide triggers hepatotoxicity. Soursop leaves have a hepatoprotective effect because they contain antioxidant compounds that can help inhibit and prevent oxidative damage to the liver. This study aims to determine the effect of soursop leaf extract on the histopathological appearance of rat liver induced by isoniazid and pyrazinamide. This research is a laboratory experimental research using design posttest only control group design. This study used 24 rats Wistar divided into four groups and given treatment for 14 days. The negative control group was not given soursop leaf extract. The positive control group was given isoniazid and pyrazinamide, the treatment group I was given isoniazid and pyrazinamide and 1 hour later continued with soursop leaf extract 72 mg/day giving soursop leaf extract 144 mg/day. The dose of isoniazid used was 189 mg/day, while the dose of pyrazinamide was 252 mg/day. Termination of rats was carried out on the 15th day to collect rat liver organs. Furthermore, histopathological examination of the rat liver was carried out. The results showed that the rats in the negative control group had normal histopathological features. In the positive control group, there was a picture of hydropic degeneration. The histopathological picture of the rat liver in the treatment group 1 showed a picture of parenchymatous degeneration and in the treatment group 2, the liver cells were normal. Administration of soursop leaf extract affected the histopathological picture of rat liver induced by isoniazid and pyrazinamide.

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1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacteria Mycobacterium tuberculosis (Kementrian Kesehatan RI, 2018). Mycobacterium tuberculosis probably still infects around a quarter of the world's population, despite the global recognition of a vaccine and the discovery of an effective four-drug treatment regimen (Drain et al, 2018). Tuberculosis is found in every country and age group and is one of the causes of death in the world. Globally in 2019 around 10 million people suffered from tuberculosis. Indonesia itself ranks 2nd in the world with the highest number of tuberculosis sufferers after India. In 2020, 351,936 cases of tuberculosis were found in Indonesia with the highest number of cases in several large provinces, namely West Java, East Java and Central Java, which accounted for almost half of the reported cases, namely 46% (Hardhana et al., 2020).

One effort to control tuberculosis is through treatment. ethambutol, isoniazid, pyrazinamide, rifabutin, rifampin, and rifapentine are some of the first-line anti-tuberculosis (OAT) drugs. streptomycin, capreomycin, cycloserine, ethionamide, amikacin, levofloxacin, moxifloxacin, and para-aminosalicylic acid are second-line OAT. The most commonly used anti-tuberculosis drug, also known as HRZE, is a combination of four drugs: pyrazinamide (PZA), isoniazid (INH), ethambutol (EMB), and rifampicin (RMP). Among these OATs, pyrazinamide and isoniazid are the drugs that

most often induce liver damage or more commonly known as drug induced liver injury (DILI) (Goh, Tee and Ho, 2020).

The liver is an important organ for humans where this organ is located between the digestive tract and circulation. The liver also plays a role in reducing exposure to toxic chemicals such as drugs (Banjuradja and Singh, 2020). Hepatotoxicity is a condition that causes liver cells to be damaged by toxic chemicals. The use of OAT regimens is one of the most common causes of hepatotoxicity (Juliarta, Mulyantari, and Yasa, 2018). The use of isoniazid itself in the presence of the CYP450 enzyme will convert metabolites from isoniazid into toxic compounds and produce free radical compounds. Meanwhile, pyrazinamide shows changes in nicotinamide levels of acetyl dehydrogenase in the liver which results in the formation of free radicals. Oxidative stress is the main mechanism of free radicals associated with anti-tuberculosis drugs which cause hepatotoxicity and damage to the liver (Djauhari, 2019).

These various conditions have caused the use of natural ingredients as medicine for various diseases to increase at this time. Plants are a source of natural ingredients that are widely used as medicine. Compounds present in plants are responsible for their activity against various diseases. Many studies have been carried out to identify active compounds in plants and determine the pharmacological action of their elements against disease. Annona muricata Linn, often called soursop, is one of them. Annona muricata Linn contains various compounds that have pharmacological actions. Acetogenin, alkaloids and flavonoids are the most active components in Annona muricata Linn. Analysis of compounds in Annona muricata Linn leaf extract shows secondary metabolites such as flavonoids, terpenoids, saponins, coumarins, lactones, anthraquinones, glycosides, tannins and phytosterols (Mutakin et al., 2022).

The body needs antioxidants as hepatoprotectors to reduce the hepatotoxic effects of drugs. Soursop leaves are an antioxidant that can reduce hepatotoxic effects. The antioxidant compounds contained in soursop leaves have a hepatoprotective effect which can help inhibit the process of free radical formation so that there is no oxidative damage to liver cells (Parapaga, Durry, Lintong, 2018).

The results of research conducted by Parapaga in 2018 found the effect of soursop leaves on preventing liver cell damage due to the toxic effects of the anti-tuberculosis drug rifampicin and showed a picture of liver cell regeneration with the administration of soursop leaf extract. In the Parapaga study, soursop leaf extract was given for 7 days at a dose of 600 mg/kgBW/day (Parapaga, Durry, Lintong, 2018). Based on the above, researchers are interested in knowing the effect of administering soursop leaf extract (Annona muricata Linn) on the histopathological picture of the liver of Wistar rats (Rattus norvegicus) induced by isoniazid and pyrazinamide.

2. METHODS

This research is an experimental laboratory research using a post test only control group design, namely a type of research that only observes the control and treatment groups after being given an action. Making extracts and raising mice was carried out at the Pharmacology Laboratory, Faculty of Pharmacy, University of North Sumatra. Preparation and examination of histopathological preparations of Wistar rats (Rattus norvegicus) were carried out at the Anatomical Pathology Laboratory at Grand Medistra Lubuk Pakam Hospital. The research was conducted starting in September.

Research Population

All members of the population, namely male white Wistar rats who met the inclusion criteria, were included in the research sample. The samples used in this study were Wistar rats (Rattus norvegicus) which met the inclusion criteria. In this study, mice were divided into four treatment groups consisting of one negative control group (-), one positive control group (+) (given isoniazid + pyrazinamide) and two treatment groups (given isoniazid + pyrazinamide and soursop leaf extract (Annona muricata L .)) with graded doses. The number of mice used as samples in this study was determined based on Federer's formula:

$$(n-1)\,(t-1) \geq 15$$

Notes:
n = number of groups

t = number of samples per group
(4 - 1) (t - 1) ≥ 15
3 (t - 1) ≥ 15
3t - 3 ≥ 15
3t ≥ 18
t ≥ 6
Sample size = n x t
= 4 x 6
= 24 mice
So for 4 groups, each group used 6 mice.

Inclusion and Exclusion Criteria

Inclusion Criteria

1. Male white rat (Rattus norvegicus strain Wistar)
2. Age 2 – 3 months
3. Body weight 150 – 200 grams

Exclusion Criteria

1. Mice that have been used in research
2. Mice got sick during the research process
3. Mice died during the research process

Research Instrument

Tools

- 4 experimental animal cages
- Sonde
- Micro pipette
- Tools for making suspensions
- Surgical tools for experimental animals
- Tools for making histology preparations
- Olympus BX 53 microscope

Materials

- Soursop leaf
- OAT Isoniazid and pyrazinamide
- Aquadest
- Experimental animal food and PAM water
- Chloroform
- Material for making histology preparations

Procedures

To get a clear picture, the research process is as follows.

1. Researchers ask for permission by arranging Ethical Clearance (EC). The researcher asked for permission to apply for research to be submitted to the educational institution, Faculty of Medicine, HKBP Nommensen University.
2. Applying for a research permit at the laboratory where the research is conducted.
3. Animal adaptation is carried out for 1 week in the animal house, where the test animals are grouped into 4 groups and each group is placed in one cage.
4. Creation of an animal model of rat liver damage.
   In producing liver damage in mice, oral isoniazid and pyrazinamide were induced with an isoniazid dose of 189 mg/day and a pyrazinamide dose of 252 mg/day, administered for 14 days. Termination was carried out on the 15th day and then surgery was carried out to see the histopathological condition of the rat's liver.
5. Making soursop leaf extract.
   Making soursop leaf extract by selecting soursop leaves that are neither too old nor too young. The soursop leaves are cleaned from dirt. The bones of the leaves are removed, washed with clean water, drained to reduce the water content and then weighed. After that, it is dried in a

drying cupboard at a temperature of ± 40°C until the soursop leaves become brittle. The dried samples were made into powder by crushing them using a blender and then stored in a dry container and tightly closed. The dry macerated powder is put into a container, then 70% ethanol solvent is added until the powder is submerged. Let stand and stir occasionally for 5 days. Next, the maserate is separated from the powder soaking, then in the same way the dregs are macerated again with 70% ethanol solvent for 2 days, and after that the macerate is separated again. The results of the macerate obtained are combined, then evaporated using a rotary evaporator at a temperature of ± 40 oC, then a thick extract is obtained (Extraction et al., 2019).

6. Calculation of the dose of soursop leaf extract.
   The dose of soursop leaf extract given in this study was based on previous research which increased the dose where the dose of soursop leaf extract with rifampin OAT was used, namely doses of 400 mg and 600 mg.
   Treatment group 1 = 800 mg x 0.018 = 14.4 mg/200g = 72 mg
   Treatment group 2 = 1600 mg x 0.018 = 28.8 mg/200g = 144 mg
   72 mg/kgBB/day and 144 mg/kgBB/day.
7. Calculation of the dose of OAT isoniazid and pyrazinamide.
   The doses of isoniazid and pyrazinamide were first converted from human to rat doses. So the human dose is multiplied by 0.018 (based on the Laurence and Bacharach table conversion).
   Human to rat dose conversion:

Dose conversion = Human dose (mg/kg) x Conversion

   The toxic dose of isoniazid for humans is 30 mg/kgBW/day. So the dose of isoniazid:
   30 mg/kg/BW x 70 kg = 2,100 mg/kgBW
   Dose conversion = 2,100 mg/kgBB x 0.018 = 37.8 mg/kgBB
   = 37.8 mg x 1,000/200
   = 189 mg
   The toxic dose of pyrazinamide for humans is 40-50 mg/kgBW/day (Djauhari, 2019).
   Then the dose of pyrazinamide:
   40 mg/kgBB x70 kg = 2,800 mg/kgBB
   Dose conversion = 2,800 mg x 0.018 = 50.4 mg/kgBB/day
   = 50.4 mg x 1,000/200
   = 252 mg
8. Process of Using.
   A total of 24 mice were divided and adapted into 4 groups. Treatment for each group is as follows:
   a) Treatment Group 1 was given food and drink but was not treated with isoniazid + pyrazinamide and soursop leaf extract.
   b) Treatment group 2 was treated with isoniazid 189 mg/kgBW/day and pyrazinamide 252 mg/kgBW/day per rat without being given soursop leaf extract for 14 days.
   c) Treatment group 3 was given treatment with isoniazid 189 mg/kgBW/day and pyrazinamide 252 mg/kgBW/day, then 1 hour later they were given soursop leaf extract at a dose of 72 mg/kgBW/day per rat for 14 days orally.
   d) Treatment group 4 was given treatment with isoniazid 189 mg/kgBW/day and pyrazinamide 252 mg/kgBW/day, then 1 hour later they were given soursop leaf extract at a dose of 144 mg/kgBW/day per rat for 14 days orally.
9. Taking rat liver samples.
   Liver samples from male white mice were taken on the 15th day. The mice were first euthanized. After that, the mice were dissected and their livers were removed and put into bottles containing 10% Neutral Buffer Formalin (NBF).
10. Procedure for making histopathology preparations.

The rat liver organ that had been placed in a bottle containing 10% Neutral Buffer Formalin (NBF) was then processed to make histopathology preparations. The specimen is cut and then placed in a cassette. The process of making preparations is carried out in several stages, starting with fixation, which is done to stop cell activity so that they do not divide and prevent cells/tissue from decaying. The fixation stage was carried out by adding 10% Neutral Buffer Formalin (NBF) for 2 hours, then 10% Neutral Buffer Formalin (NBF) solution for 1.5 hours. After the fixation stage, it is followed by the dehydration stage which aims to remove water from the tissue using graded concentrations of alcohol over a certain time using four alcohol concentrations, namely 70% alcohol, 96% alcohol, 96% alcohol and absolute alcohol (100%). The purpose of using graded concentrations of alcohol in this process is to gradually remove water from the sample. The clearing stage is carried out to clean remaining alcohol from the tissue, clearing is carried out with xylol I for 1 hour and xylol II for 1.5 hours. The next stage is impregnation which aims to remove xylene fluid (clearing agent) from the tissue and replace it with paraffin. The stages of the dehydration, clearing and impregnation process are presented in the table.

Table 1. Estimated time for the dehydration, clearing and impregnation processes

Process	Time
NBF 10%	2 hours
NBF 10%	1,5 hours
Dehydration	
Alcohol 70 %	1,5 hours
Alcohol 96 %	1,5 hours
Alcohol 96%	1,5 hours
Alcohol 100%	1,5 hours
Alcohol 100%	1,5 hours
Alcohol 100%	1,5 hours
Clearing	
Xylol	1 hour
Xylol	1,5 hours
Impregnation	
Paraffin	1,5 hours
Paraffin	2 hours

Next is the embedding process, namely the process of casting the sample using a mold with paraffin. This is done to make the cutting process easier. The preparations were cut to a size of 4 µm using a microtome. The sample pieces were then stretched by first being placed in a water bath containing water at a temperature of 49°C. After that, the sample is placed on a glass slide. The glass slide is then placed on a hot plate so that the sample on the slide dries.

The next stage is staining so that observation of cells or tissue becomes easier. Sample staining was carried out using hematoxylin and eosin dyes. After the coloring process is complete, the next stage is to cover with cover glass (mounting) using entellan which is an adhesive liquid to attach the cover glass to the glass preparation. This is done so that the refractive index is flat so that there are no difficulties when observations are made (Rahmawanti, Nur, and Mukhlis, 2021).

11. Examination of histology preparations.

Rat liver damage was examined by observing histology preparations using an Olympus BX53 microscope with 400x magnification, then the visual field was examined and the changes that occurred were determined using the Manja Roenigk Histopathology Scoring.

Table 2. Criteria for assessing the degree of histopathology of liver cells Manja Roenigk Histopathology Scoring Model

Rate of Change	Score	Criteria
Normal	1	• Round cells • Intact cytoplasm • The cell membrane is not damaged

Parenchymatous Degeneration	2	• The cell nucleus is round • Cell swelling • Cytoplasm is cloudy
Hydropic Degeneration	3	• Cytoplasm undergoes vacuolization • Vacuoles appear clear • Cytoplasm pale • Cells appear enlarged • The nucleus is in the center
Necrosis	4	• The nucleus shrinks (pyknosis) • The nucleus breaks into fragments (karyokinesis) • Nucleus lysis(karyolysis) • Cell membrane lysis

After the observation is complete, the results of the observation assessment based on the parameters are recorded and arranged in table form. The data was then tested for significance regarding the effect of the treatment group with the help of the SPSS program.

3. RESULTS AND DISCUSSION

This research was conducted to see the effect of giving soursop leaf extract on the histopathological picture of the liver of Wistar rats Rattus norvegicus induced by isoniazid and pyrazinamide. Histopathological observations were carried out by looking at liver cell damage in the entire field of view per slide. Each preparation was read using an Olympus BX 53 digital microscope where observations and assessments were carried out by an Anatomic Pathology specialist.

The liver is an excretory organ whose function is to detoxify toxic substances so that liver damage is an indication of whether a substance is toxic or not. If the liver is continuously exposed to drugs and chemicals in the long term, the cells in the liver can experience changes, especially in hepatocytes, such as fatty degeneration and necrosis which can reduce the ability of cell regeneration, causing permanent damage to cell death (Sijid et al, 2020) Hepatocyte damage was measured for microscopic changes using the Manja Roenigk scoring system, namely normal, parenchymatous degeneration, hydrophic degeneration and necrosis.

In the negative control group, a normal histopathological picture was found, namely round cells with round, non-dense cell nuclei, purple and intact cytoplasm and no visible damage to the cell membrane, the central veins and sinusoids still appeared intact and in normal condition (Muhartono, Oktarlina, and Purohita, 2019). This is in line with research conducted by Windi et al in 2018, in the negative control group which was only given food, a picture of liver cells that were not damaged was visible. The cells are round, have intact and purple cytoplasm, the cell membrane is not damaged and the cell nucleus is round and not solid (Sari, Saebani, and Dhanardhono, 2018).

In the positive control group, the histopathological picture of hydropic degeneration was found, namely that the cytoplasm was vacuolized, the cytoplasm was pale, the cells appeared enlarged, but in fact there was a slight visible cell necrosis. Hydropic degeneration can occur due to secondary responses such as toxins, free radicals, viruses or bacteria. Degeneration in liver cells is thought to be due to disruption of the sodium-potassium pump in the cell membrane due to peroxidation of membrane lipids, causing hypernatremia in the cells which causes water to enter. Necrosis is an injury condition resulting from irreversible death of cells in living tissue due to the presence of toxic substances and radioactive rays. Several other things, such as lack of blood supply, absence of nerve innervation, temperature and mechanical trauma, can also cause tissue necrosis. Before necrosis occurs, the nucleus will first become pyknotic, which is characterized by the color of the cell nucleus appearing darker when compared to normal liver cell nuclei. This happens because the chromosomes in the pyknotic nuclear cells experience homogenization and absorb a lot of dye (Yana and Budijastuti, 2022). In hydropic degeneration, there are many vacuoles in the cytoplasm and the size of the cells also looks larger than normal cells. The vacuoles appear swollen and the cells involved are paler in color.

In treatment group 1, the histological picture showed that most of them had parenchymatous degeneration, but in some parts the liver appeared normal. Microscopically, the size of the cells is swollen and the cytoplasm looks more turbid than normal cells. In the cytoplasm, granules appear that look like dots because they contain more water due to the cell's inability to maintain fluid and ion homeostasis. Degeneration is formed due to the presence of a toxic substance that disrupts cell organelles, mitochondria and the endoplasmic reticulum so that the cell oxidation process does not proceed well. This causes cells to be unable to eliminate metabolite products, causing accumulation of water (Muhartono, Oktarlina, and Purohita, 2019). However, in some parts the liver appears normal.

In treatment group 2, the histopathological picture appeared normal with round cells with round cell nuclei, central veins and sinusoids that appeared intact, but in some parts parenchymatous degeneration was still visible. This picture shows an improvement in the histopathological appearance of the liver tissue of mice given soursop leaf extract. In the results of microscopic examination, there were differences in histopathological features between treatment groups. The group given soursop leaf extract along with isoniazid and pyrazinamide induction showed a picture of cells undergoing regeneration. This shows the effect of giving soursop leaf extract in preventing damage to liver cells due to the toxic effects of using the anti-tuberculosis isoniazid and pyrazinamide as well as triggering the regeneration of liver cells.

The results of this research are in line with research conducted by Parapaga in 2018. Parapaga conducted research by inducing rifampin on mice, and then comparing the effect of soursop leaves on the histopathological picture of mice. Parapaga found that microscopic images of the liver cells of mice that were induced by rifampicin and then given soursop leaf extract the following week showed improvement. In the group that was not given soursop leaf extract, the microscopic image of the mice's liver cells was mostly necrotic, while in the group that was given soursop leaf extract, cells were seen undergoing regeneration (Parapaga, Durry, and Lintong, 2018). An increase in the number of normal hepatocyte cells is possible due to the administration of soursop leaf extract which contains antioxidants.

Antioxidants are substances that are able to defend cells from fragility and are able to repair damaged cells. Antioxidants are important compounds that function as scavengers of free radicals (Merdana et al., 2019). One compound that can act as an antioxidant is flavonoid compounds. Flavonoid compounds can protect body cells from oxidative damage so that cell membranes can function properly. Flavonoids as antioxidants work by capturing free radicals. Flavonoid compounds are one of the substances contained in soursop leaves. The presence of flavonoids in soursop leaves causes soursop leaves to play a role in inhibiting damage to liver cells so that cells can regenerate and tissue improves (Rarangsari, 2015).

4. CONCLUSION

Based on data analysis and findings obtained from the previous session, the researchers provided several conclusions from this study, namely: giving soursop leaf extract had an effect on the histopathological picture of rat liver induced by isoniazid and pyrazinamide. Then, increasing the dose of soursop leaf extract had a better effect on the histopathological picture of rat liver induced by isoniazid and pyrazinamide.

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