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Evaluation of The Hepatoprotective Effect of *Plantago major* Extract in A Rifampicin-Isoniazid Induced Hepatitis Rat Model

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Abstract

Tuberculosis (TB) is an ancient human disease caused by *Mycobacterium tuberculosis* which affects the lungs, making pulmonary disease the most common presentation. Herbal medicine began to be developed as hepatoprotection. The hepatoprotection effects of *Plantago major* extract on serum glutamic pyruvic transaminase (SGPT), liver tissue Malondialdehyde (MDA), and histopathological changes were evaluated in rifampicin and isoniazid-induced hepatitis rats. Thirty male Wistar rats were divided into 6 groups: Group I received 1 % PGA, with hepatitis induced in the remaining groups by rifampicin and isoniazid 50 mg/kg BW/day, group II was only given rifampicin and isoniazid, group III was given curcuma 10.8 mg/kg BW/day and groups IV (PM1), V (PM2), and VI (PM3) were given *Plantago major* extract at a dose of 20.3, 40.5, and 81 mg/kg BW/day, respectively. The rats were treated for 28 days. Administration of *Plantago major* extract (20.3 and 40.5 mg/kg BW/day) inhibited the elevation of serum SGPT and MDA levels, with less portal inflammation than the negative control group. The rats treated with the higher dose of 81 mg/kg BW/day had serum SGPT, MDA, and percentage portal inflammation equivalent to the negative control group. The *Plantago major* extract at a dose of 20.3 and 40.5 mg/kg BW can inhibit the elevated serum SGPT. *Plantago major* extract exerts dose-dependent hepatoprotection effects in a rifampicin-isoniazid induced hepatitis rat model by reducing elevated levels of SGPT, liver tissue MDA, as well as the percentage of portal inflammation.

Keywords: Antituberculosis Drugs, Hepatoprotection, Histopathology, Isoniazid, MDA, *Plantago major*, Rifampicin, SGPT

Introduction

Tuberculosis (TB) is a global public health problem; however, the prolonged treatment and multidrug use of current first-line anti-TB drugs can cause side effects such as hepatitis [1]. Despite the gains in tuberculosis control and the decline in both new cases and mortality, TB still accounts for a huge burden of morbidity and mortality worldwide. The bulk of the global burden of new infection and tuberculosis death is borne by developing countries with 6 countries, India, Indonesia, China, Nigeria, Pakistan, and South Africa, accounting for 60 % of TB death in 2015 [2]. This condition not only increases morbidity and mortality but also has the potential to lead TB patients to stop or discontinue treatment, thereby increasing the risk of multidrug-resistant tuberculosis (MDR TB). Shang *et al.* [3] reported that TB patients with anti-TB drug-induced hepatitis had a 2.11 times higher risk of needing extended treatment and 9.25 times higher risk of failing therapy than TB patients without anti-TB drug-induced hepatitis.

Morbidity and mortality due to TB is a fundamental problem due to side effects caused by anti-TB drugs. The most serious side effect of anti-TB drugs is hepatotoxic. The 3 drugs for TB such as isoniazid, rifampicin and pyrazinamide are hepatotoxic. Hepatotoxicity from anti-TB drugs is most common in the first 2 months of treatment. Hepatotoxicity is classified into 2 types of reactions: Intrinsic (predictable) and idiosyncratic (unpredictable). Hepatotoxicity due to anti-TB drugs causes extensive and permanent

liver injury and can cause death if not detected in the initial stage. In addition to hepatotoxicity, anti-TB drugs can also cause cirrhosis, liver cancer, and lead to death [4]. The most severe side-effects leading to interruption of treatment were hepatotoxicity (11 %), skin rash (6 %), and joint pain (2 %) [5].

This suggests the need of preventing those side effects to ensure the treatment is successful, such as administering hepatoprotective compounds. *Plantago major* is herbal plant with antioxidant and hepatoprotective properties [6-9], rich in phenol and flavonoid compounds which function as antioxidants [6]. Phenol compounds like acetonide [8] and flavonoids like luteolin [10,11], apigenin [12,13], baicalein [14,15], and hispidulin [16] have antioxidant and hepatoprotective properties, thereby protecting against free radical damage [8,17]. The 5 active compounds in *Plantago major* are considered to have good antioxidant properties. According to Mohamed *et al.* [18], the presence of phenol and flavonoids compounds are most common in ethanol extracts made from the plant leaves. In this study, MDA and SGPT are selected as parameters with different purposes. SGPT is used as a parameter for liver injury, while MDA is used to assess whether liver damage is caused by oxidative stress pathway. Considering SGOT distribution is more common in the heart than in liver, authors use SGPT markers to ensure that the findings more accurately reflect liver injury [19,20]. There is a lack of research evaluating the hepatoprotective effect of *Plantago major* in an anti-TB drug-induced hepatitis animal model, therefore, this study evaluated the hepatoprotective effect of *Plantago major* extract in a rifampicin and isoniazid-induced hepatitis animal model.

Materials and methods

Chemicals and reagents

Plantago major leaves were obtained from Taman Penelitian Manoko, Lembang. Rifampicin 600 mg (Kimia Farma, Jakarta), isoniazid 300 mg (Kimia Farma, Jakarta), and curcuma 20 mg (curcuma FCT, Soho, Jakarta) were purchased from pharmacies in Bandung. Commercial kits from RANDOX Laboratories Ltd (Cat. No. AL 1205) and Bio-assay System (Cat. No. DTBA-100) were used for SGPT and MDA tests.

Preparation of *Plantago major* leaf extract

Plantago major leaves were cleaned from dust and dried at room temperature, then crushed using a blender. The leaves were macerated using 70 % ethanol with b/v (1/5 g/mL) for 24 h and the process was repeated 3 times. The solvent was evaporated by a rotary evaporator. From 1.8 kg of fresh leaf weight, 475.6 g of dried leaves were collected yielding 111.3 g of ethanol *Plantago major* extract.

Experimental animals

This research received ethical approval from the Komisi Etik Penelitian Universitas Padjadjaran Bandung (Ref no: 1096/UN6.KEP/EC/2020). Thirty healthy male Wistar 12-week-old rats weighing 200 - 250 g was obtained from the Laboratorium Biofarma, Bandung. The rats were acclimatized for 10 days in a quiet room in a 12-h light/dark cycle with ad libitum access to food and drink. The animals were then separated into 6 groups, with group I (baseline) rats receiving only 1 % PGA and hepatitis induced in the other groups using rifampicin (RIF) and isoniazid (INH) in a dose of 50 mg/kg BW/day. Rats in group II (negative control) were only given RIF-INH, group III (positive control) were given RIF-INH + Curcuma 10.8 mg/kg BW/day, group IV (PM1), V (PM2), and VI (PM3) were given RIF-INH + *Plantago major* extract at a dose of 20.3, 40.5 and 81 mg/kg BW, respectively. The rats were treated for 4 weeks and on day 29, the rats were sacrificed, and blood was collected to quantify serum SGPT, and liver tissue was processed to quantify MDA levels and histopathology examination.

Measurement of Serum Glutamic Pyruvic Transaminase (SGPT) level

The SGPT test was performed using a colorimetric method with the ALT kit from RANDOX Laboratories Ltd. (Cat. No. AL 1205). Briefly, 3 mL of blood was drawn directly from the heart and the serum was separated by centrifugation (15 min at 3,000 rpm) and stored at −20 °C until analysis.

Measurement of Malondialdehyde (MDA) level

MDA was quantified using a colorimetric method with the kit from Bio-assay System (Cat. No. DTBA-100). Liver tissue (20 mg) was homogenized in cold PBS, then 100 µL of tissue lysate was transferred to a new Eppendorf tube containing 100 µL of 10 % TCA and incubated 5 min on ice. The supernatant was collected by centrifugation (14,000 rpm for 5 min) and transferred to a new Eppendorf

tube containing 200 µL of TBA reagent, vortexed, and incubated at 100 °C for 60 min. The sample was cooled to room temperature and the color change was measured using a spectrophotometer at 550 nm.

Histopathology examination

The liver tissue was divided into the left and the right lobes, with each lobe undergoing a transverse incision to separate the superior, medial, and inferior sections resulting in 6 samples of tissue per rat. The tissue samples were fixed in a 10 % formalin for 6 h before being cut into 5-µm sections, fixed in paraffin, and stained with Hematoxylin Eosin (HE). The number of portal areas that had inflammation or not, up to 30 areas, were then counted and the percentage of the portal inflammatory area was calculated by dividing the number of areas that had inflammation by the total area (30) multiplied by 100 %.

Data analysis

SGPT, MDA, and histopathological alterations were compared using one-way ANOVA followed by Tukey HSD with a 95 % confidence level and a significant level of < 0.05 using SPSS 17.0 software [21,22]. The diagrams were created using GraphPad Prism 8 [23,24].

Results and discussion

Serum Glutamic Pyruvic Transaminase (SGPT), liver tissue malondialdehyde (MDA), and histopathological changes were evaluated to assess the extent of hepatocellular injury in this study. The administration of isoniazid and rifampicin at a dose of 50 mg/kg BW/day intragastrically for 28 days induced hepatitis in this study as evidenced by elevated SGPT and MDA levels. **Table 1** showed that RIF-INH+ PM1 decreased SGPT level significantly (4.481 U/L) than RIF-INH (9.950 U/L). Meanwhile, RIF-INH+PM2 has better activity for decreased MDA level (1.843 µM) than RIF-INF (2.930 µM). The result of the histopathology changes in rat rifampicin-isoniazid induced could increase histopathology score. All treatments in this study were able to reduce histopathology scores in rifampicin-isoniazid induced rat.

Table 1 The effect of the *P. major* extract on SGPT serum, liver tissue MDA, and histopathology changes in the rifampicin and isoniazid-induced hepatitis rat model.

Group	SGPT (U/L)	MDA (µM)	Histopathology
I: Baseline	6.093 ± 1.372	1.616 ± 0.259	20.6 ± 4.9
II: RIF-INH	9.950 ± 1.897*	2.930 ± 0.520**	64.2 ± 5.0**
III: RIF-INH + Curcuma	7.508 ± 1.661	2.026 ± 0.271†	37.3 ± 4.3*††
IV: RIF-INH + PM1	4.481 ± 0.481†	1.896 ± 0.366†	40.0 ± 3.8*††
V: RIF-INH + PM2	5.259 ± 0.635†	1.843 ± 0.451††	43.0 ± 3.0**††
VI: RIF-INH + PM3	7.987 ± 2.441	2.777 ± 0.286*$^{\circ}$	49.2 ± 4.2**

*: Significant difference with $p < 0.05$ compared to baseline.
**: Significant difference with $p < 0.01$ compared to baseline.
†: Significant difference with $p < 0.05$ compared to negative control.
††: Significant difference with $p < 0.01$ compared to negative control.
$^{\circ}$: Significant difference with $p < 0.05$ compared to positive control.

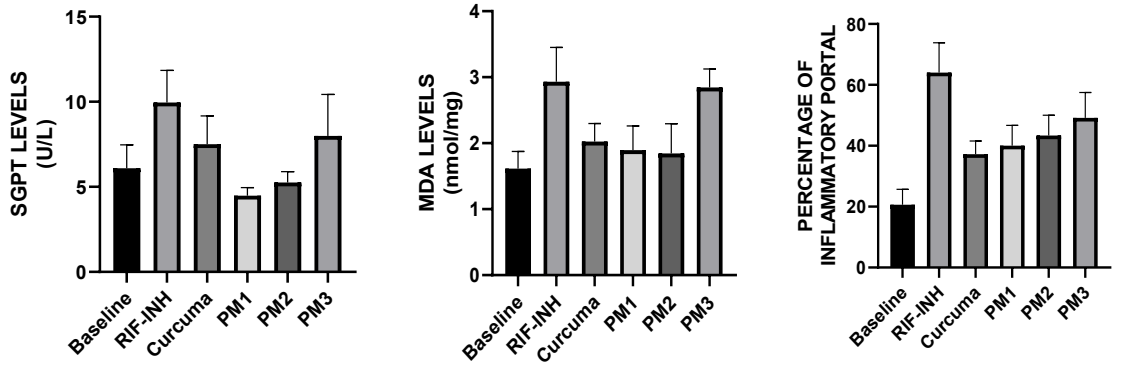
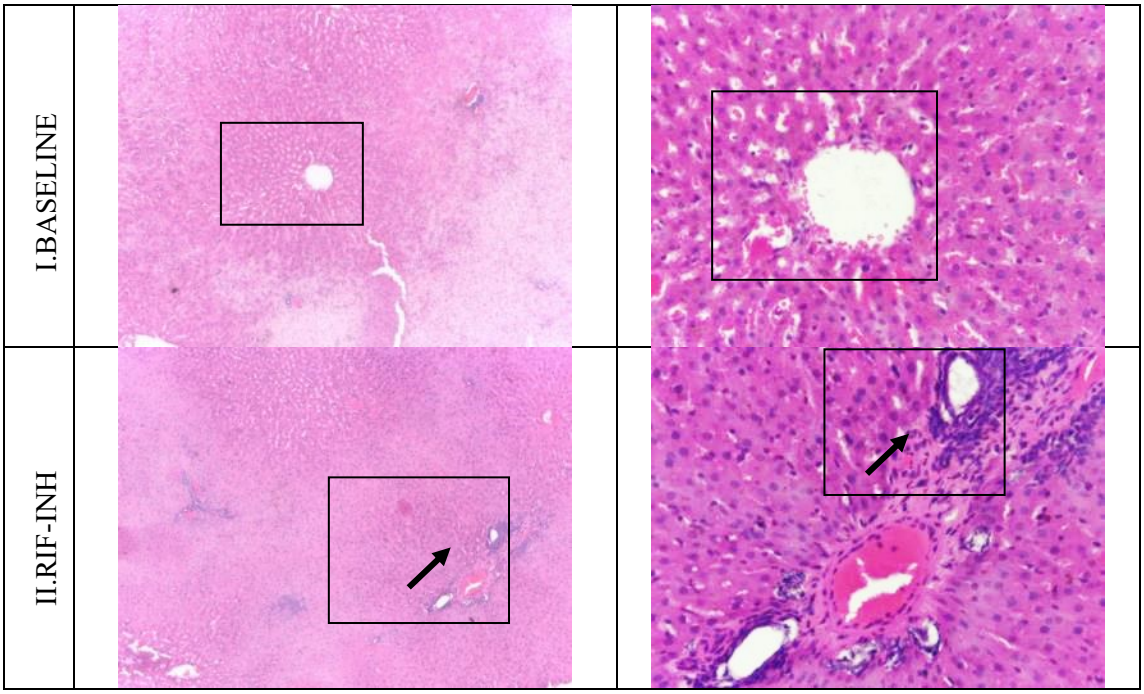


Figure 1 The effect of the *P. major* extract on SGPT serum, liver tissue MDA, and histopathology changes in the rifampicin and isoniazid-induced hepatitis rat model.

MDA is a product of lipid peroxidation that can be used as a biomarker of oxidative stress [25,26] MDA can be obtained from tissue or serum samples and in this study, the MDA was quantified in liver tissue samples so that the results represented the specific oxidative stress processes occurring in the liver.

SGPT is an enzyme found in the cytoplasm of liver cells, which will exit the cells into the bloodstream if there is a liver injury, hence an increase in SGPT levels in the blood indicate liver cell damage. SGPT is found in various cells, but the liver contains the majority of the SGPT in the body [27]. SGOT is another potential biomarker most found in the heart, but SGPT was used as a biomarker in this study so that the results are more specifically representative of liver injury. Hepatocellular injury was further confirmed by histopathological changes assessing the percentage of the inflammatory portal area (**Figure 2**). Portal inflammation is morphological disorder that arises due to the administration of isoniazid [28,29].



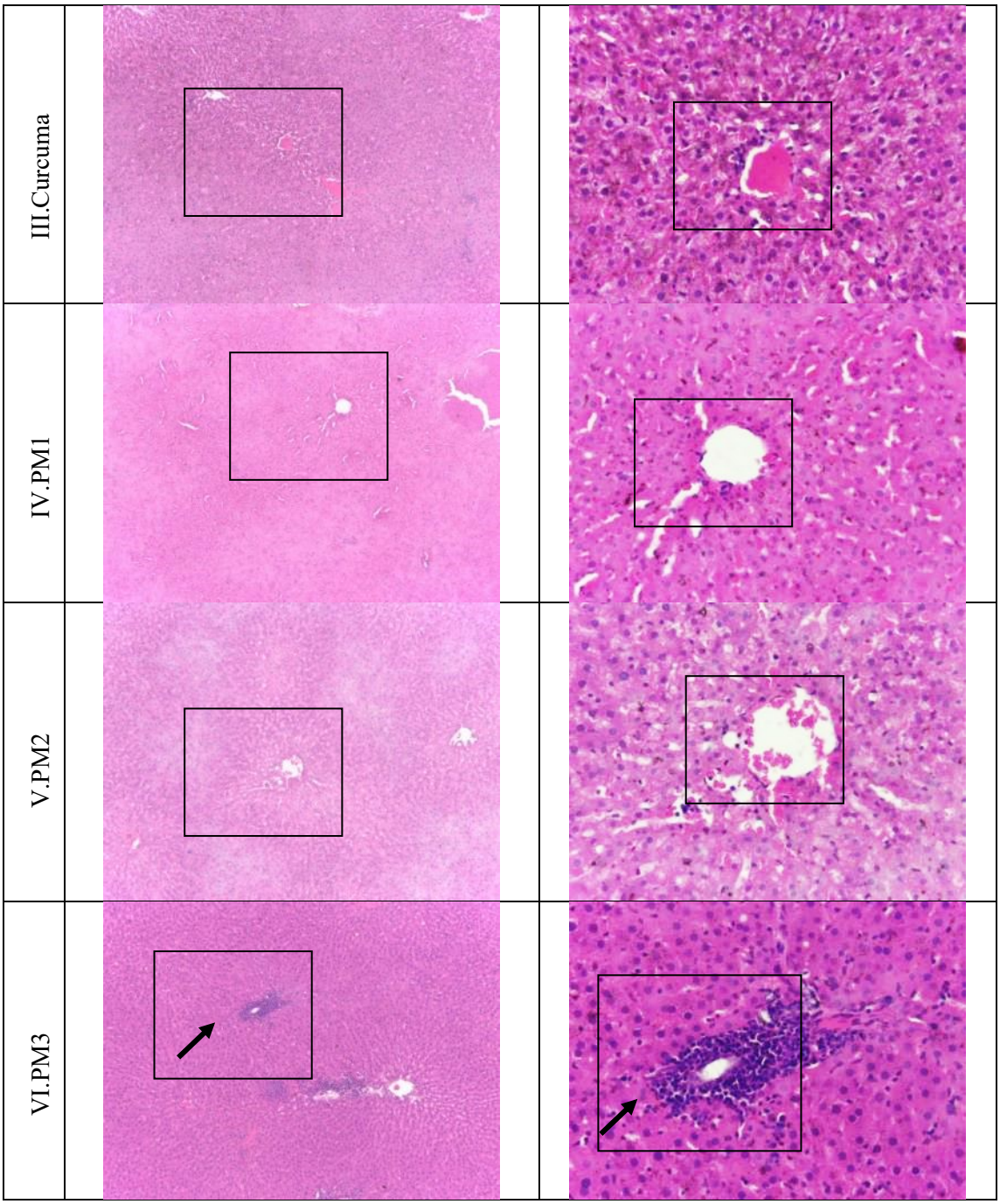


Figure 2 Histopathological changes in the liver tissue samples using HE stain (left side = original magnification 10×; right side = original magnification 40×). Portal inflammation is indicated by the presence of many inflammatory cells in the portal vein area (→).

Rifampicin and isoniazid are the first-line anti-TB drug combination most associated with hepatitis side effects [30]. Hepatitis is caused by the formation of reactive metabolites, which are the result of isoniazid metabolism that trigger lipid peroxidation, causing damage to the liver tissue. Furthermore, the metabolite form of isoniazid (acetyl hydrazine) can bind covalently to macromolecules in the liver, generating a neoantigen that causes further damage due to inflammatory processes via antibody-dependent cytotoxicity. Injured hepatocytes release TGF-β cytokines, which, in combination with reactive metabolites and lipid peroxidation products (MDA and HNE), activate Kupffer cells and stimulate other

inflammatory cells, such as neutrophils, to migrate and release oxidant products, thereby worsening the severity of the injury and resulting in a hepatocellular injury cycle [31,32]. Rifampicin has a potential effect on isoniazid metabolism, acting as a CYP450 inducer, causing the reactive metabolite isoniazid to generate increasingly more products [1].

Curcuma had an inhibitory effect on increased MDA levels but did not affect increased SGPT levels, with a lower percentage of portal inflammation in the treated rats than in the negative control rats (**Figures 1** and **2**). This shows that Curcuma could protect the liver against rifampicin and isoniazid-induced hepatitis. According to Farzaei *et al.* [33], the phenol group in Curcuma has antioxidant as well as anti-inflammatory properties. Curcuma acts on several molecular pathways, including the ERK/p38/MAPK pathway, Nrf2/ARE/Keap1 pathway, improved detoxification gene expression regulation, and decreased regulation of Rac1, NOX1, and transduction of Rac1-GTP [28]. Curcuma is commonly used in a variety of doses. In the present study, rats were given a dose of 10.8 mg/kg BW/day, which was a conversion from a human dose of 120 mg/day [34]. Adewale *et al.* [35] used a higher Curcuma dose of 20 mg/kg BW in rats, showing that it had hepatoprotective effects in rats with sodium nitrite-induced hepatitis, while Singh and Sharma [36] reported that a Curcuma dose of 200 mg/kg BW demonstrated higher hepatoprotection effects in lindan-induced hepatitis rat models. A range of Curcuma doses have been reported and according to the NCI, Curcuma doses of 847-959 mg/kg BW are NOAEL (No Observable Adverse Effect Level) in genotoxic experiments on rats [37,38].

In this study, *Plantago major* extract was given along with rifampicin and isoniazid induction, as it is considered a hepatoprotection to prevent further hepatocellular injury. Furthermore, this approach is more clinically relevant, as patients with tuberculosis are unlikely to delay the administration of their anti-TB drugs simply because they must consume *Plantago major* first. *Plantago major* can also be given as a supplement therapy and administered concurrently with the beginning of anti-TB therapy in a clinical setting.

The administration of extracts PM1 and PM2 showed hepatoprotection effects, as evidenced by the inhibition of elevated levels of SGPT, MDA, and a lower percentage of portal inflammation than the negative controls (**Figures 1** and **2**). This is in line with the hypothesis that *Plantago major* contains bioactive phenolic substances including acetonide and flavonoid substances like luteolin, apigenin, baicalein, and hispidulin, which have antioxidant and hepatoprotective properties [6]. Several other researchers who used other herbals with those bioactive properties also reported hepatoprotective effects. The administration of the extracts PM1 and PM2 also had hepatoprotective effects equivalent to Curcuma, if not better, as Curcuma only inhibited the elevation of MDA levels, whereas extracts PM1 and PM2 inhibited the elevated SGPT and MDA levels. Although the bioactive compounds of *Plantago major* and Curcuma are different, both substances have phenolic groups that function as electron donors, so they may exert their hepatoprotective effects via a similar mechanism, such as, via NF-κB, which is important for the synthesis of pro-inflammatory cytokines [8,10,13,15,16].

The PM3 had a toxic effect on rats, as evidenced by the increased MDA levels and percentage portal inflammation in the PM3 group like the negative control group (**Figures 1** and **2**). Guil *et al.* [39] reported that *Plantago major* containing erusit acid has potentially toxic effects if consumed in massive quantities and Bozcali *et al.* [40] found that erusit acid increased the cardiotoxic effect in rats given doxorubicin. More research is needed to assess the effects of PM3 extract on other biomarkers and organs as well as drug interactions with rifampicin and isoniazid.

This study has some limitations. There was no published effective dose of ethanol extract of *Plantago major* in previous trials, thus the standard from the National Agency of Drug and Food Control of Indonesia, which is based on the weight of fresh *Plantago major* used daily (7.5 g daily) in boiled water, was used in this study. Although human doses were converted to rats in this study, the extraction process was conducted using ethanol, which may affect the quantity and quality of the samples. Therefore, further research is needed to determine the effective dose that produces the most optimal hepatoprotection effect. Also, the MDA quantification method used in this study (TBARS method) is a conventional method and extremely sensitive but other methods such as HPLC or ELISA may increase the sensitivity and specificity of the quantification [40]. There are also more stable biomarkers, such as 4-hydroxynonenal (4-HNE) and isoprostane that can be used in lipid peroxidation tests [26,42]. However, due to a limitation of time and resources, the investigators used a commercially available kit-based conventional method. The degree of portal inflammation was assessed based on the presence of inflammatory cells and this method does not distinguish the quality of inflammation in each portal area.

Conclusions

Plantago major extract exerts dose-dependent hepatoprotection effects in a rifampicin-isoniazid induced hepatitis rat model by reducing elevated levels of SGPT, liver tissue MDA, as well as the percentage of portal inflammation.

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