BUKTI KORESPONDENSI

ARTIKEL INTERNASIONAL BEREPUTASI

Judul Artikel	:	***Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats**
Jurnal	:	Tropical Journal of Natural Product Research (TJNPR)
Penulis	:	Lusi P. Dwita, **Ni Putu Ermi Hikmawanti***, Juhairoh Husniah, Muhammad Hazraj, Siti N. Bela, Eva Aryanti

No.	Perihal	Tanggal
1.	Bukti *submit* dan artikel yang di-*submit*	12 Agustus 2022
2.	Bukti artikel diterima	22 Agustus 2022
3.	Bukti hasil *review* dan file komentar *reviewer*	24 Agustus 2022
4.	Bukti hasil revisi dan artikel yang direvisi	25 Agustus 2022 & 7 Oktober 2022
5.	Bukti artikel proses *galley proof* dan artikel yang dikoreksi *galley proof*	25 Desember 2022
6.	Bukti artikel telah terbit *online* pada website Jurnal	30 Desember 2022

1. Bukti *submit* dan artikel yang di-*submit* (12 Agustus 2022)

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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id>

Manuscript acknowledgement

1 message

Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Fri, Aug 12, 2022 at 5:29 PM
To: ermy0907@uhamka.ac.id

Dear Dr Hikmawanti,

Thank you for submitting your original manuscript to the Tropical Journal of Natural Product Research (www.tjnpr) https://www.scopus.com/sourceid/21100933230 SCOPUS , published by the University of Benin and Natural Product Research Group.

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The peer-review process will commence immediately, as the manuscript will be passed to an editor for initial assessment as soon as possible.

Title: ***Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Rats**

Best regards

Abiodun

Professor Abiodun Falodun, PhD

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Editorial decision on manuscript submitted for publication in TJNPR

2 messages

Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Mon, Aug 22, 2022 at 11:48 PM
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Dear Dr. Hikmawanti,

The manuscript submitted to the Tropical Journal of Natural Product Research www.tjnpr.org https://www.scopus.com/sourceid/21100933230 has been carefully reviewed by competent experts.

Find attached the details of the decision.

Please send your response urgently to the Editor-in-Chief, to enable us to process your manuscript for the next issue **Vol 6 issue 8, 2022**.
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Title: *Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Rats

Authors: Lusi Putri Dwita, Ni Putu Ermi Hikmawanti*, Juhairoh Husniah, Muhammad Hazraj, Siti Nur Bela, Eva Aryanti

TJNPR Editorial Decision: accepts with moderate corrections

Congratulations

Best regards

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Professor Abiodun Falodun, PhD

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Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. DR. HAMKA (UHAMKA), East Jakarta, Indonesia 13460

Dear Dr. Hikmawanti,

Provisional Acceptance letter for Article Manuscript Number TJNPR AUG102ARN

Title: *Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Rats

Authors: Lusi Putri Dwita, Ni Putu Ermi Hikmawanti*, Juhairoh Husniah, Muhammad Hazraj, Siti Nur Bela, Eva Aryanti

I am pleased to inform you that your paper sent to the Tropical Journal of Natural Product Research has been reviewed and recommended for publication as a research article.

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Review comments

11 messages

Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Wed, Aug 24, 2022 at 2:34 PM
To: ermy0907@uhamka.ac.id

Review comments (***Plectranthus scutellarioides*** **Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Rats)**

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Begin abstract with a brief background.

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A declaration of the liability of the authors for claims relating to the content of this article should also be included when submitting the revised manuscript. This should be stated as follows;

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Best regards

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The Editorial Team of the Tropical Journal of Natural Product Research kindly request you to review the enclosed article. Please complete the form and return to the Editor-in-Chief, editor.tjnpr@gmail.com; editor.tjnpr@uniben.edu

A. MANUSCRIPT

Journal	**Tropical Journal of Natural Product Research**
Manuscript Number	TJNPR AUG 102 AR
Type of paper	
Title of paper	***Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Rats**
Name of Authors	

B. REVIEWER'S SPECIFIC COMMENTS PER SECTION OF MANUSCRIPT

Abstract	Effect the corrections as indicated .
Introduction	Ok. Effect the corrections as indicated
Methodology	The methods chosen are suitable and adequate for the study. Use the chemical name instead of the brand name of the reference drug. Explain how the animals were sacrificed. Effect the corrections as indicated.
Results	Separate the figures and tables of results from the results presentation section. Effect the corrections as indicated.
Discussion	Ok. Effect the minor corrections.
Conclusion	Ok. The conclusion is supported by the results.
References	Ok. Adequate and current
Figures, Tables	Separate the figures and tables of results from the results presentation section

C. REVIEWER'S GENERAL COMMENTS AND REMARKS

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D. REVIEWER'S RECOMMENDATION

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E. REVIEWER'S INFORMATION

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Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

ABSTRACT

~~A high incidence of drug-induced liver injury leads to an increase in chronic liver disease cases worldwide. Therefore, it is essential to find a new potential candidate as a hepatoprotector to prevent the disease.~~ This study ~~aimed to~~ determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Legalon®). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Legalon®. The ~~given~~ data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

Keywords: Liver injury, *Plectranthus scutellarioides*, antioxidant

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Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver [1]. A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity [2]. The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage [3]. Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipta alba*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Mimosa pudica*, and *Silybum marianum* [4], where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk [5], as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities [6,7]. The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-O-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-O-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity [8]. This study ~~purposed to~~ evaluated whether *P. scutellarioides* could ~~be applied to~~ reduce liver damage by isoniazid and rifampicin ~~drugs~~.

Materials and methods

P. scutellarioides leaves Extraction

The leaves ~~of *P. scutellarioides*~~ were obtained from Balitro, Indonesia and identified and authenticated as~~. The determination result in the plant species is~~ *Plectranthus scutellarioides (L.) R. Br.* from the family *Lamiaceae*. A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated ~~soaked~~ for 24 hours. The maceration was done seven times with new~~renewed~~ solvent. The filtrate~~macerate~~ was evaporated in a vacuum rotary evaporator and then continued with a water bath to obtain the thick extract.

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Determination of P. scutellarioides Leaves Extract Water Content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of Loss Drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Alkaloid Identification

0.5 g thick extract, added with 8 ml of 2N HCl, then heated, cooled, and strained. The filtrate was divided into four parts: the first tube was blank, and the second part was added with Bouchardat reagent, where blackish-brown deposits indicate the presence of alkaloids. In the third tube, the Dragendorff reagent was added, where the formation of brown-orange precipitate shows the presence of alkaloids, and in tube 4, a Mayer reagent was added, where a yellowish-white precipitate indicates the presence of an alkaloid (Ministry of Health Republic of Indonesia, 2008).

Flavonoid Identification

0.5 g thick extract was added with 2 mL of ethanol, heated then filtered. Then ten drops of concentrated HCl and 0.1 g of Mg metal were added to the filtrate. The red color indicates the presence of flavonoids.

Phenolic Identification

0.5 g of thick extract was put into a test tube and then added with 2 mL of ethanol, then 2-3 drops of 5% $FeCl_3$. The formation of a color change from the initial color of light green to black-blue indicates the presence of phenolic.

Saponin Identification

0.5 g of thick extract plus 10 mL of hot water, cooled and shaken vigorously for 30 seconds. The presence of saponin was indicated by 1-10 cm froth formation, which lasted for 10 seconds, and the foam did not disappear after being added with one drop of HCl 2N.

Tannin Identification

0.5 g of thick extract was added with 15 mL of water, homogenized, transferred into a test tube, boiled for several minutes, and then filtered. The filtrate was added with 10% gelatine and the formation of white deposits indicates the presence of tannin.

Identification of Triterpenoids and Steroids

0.5 g of thick extract was added with 5 mL of ethanol, heated, then filtered and cooled. The filtrate was evaporated and then added with three drops of ether, three drops of anhydrous acetic acid, and one drop of concentrated sulfuric acid. The formation of the red color indicates the presence of triterpenoids, and the formation of the green color indicates the presence of steroids.

Determination of Total Flavonoid Levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

$$Total\ Flavonoid = \frac{Flavonoid\ consentration\ (\mu g/mL) \times sample\ volume\ (mL) \times dilution\ factor}{Extract\ weigh}$$

Animals and experimental design

Wistar rats (150-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25° C and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control ~~(given carrier) ,~~ and positive control (Legalon®). All administration was done orally, ~~The test material was given p.o~~ 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg p.o orally every day for 28 days.

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Commented [CFP6]: Use the chemical name

Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells [10], were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with -1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloracetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbiturate (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results

Plectranthus scutellarioides leaves extract characteristics

Ethanol extract ~~of 70%~~ of *P. scutellarioides* leaves was obtained in the form of concentrated blackish-brown extract and a slightly bitter taste ~~(with characteristics presented in~~ Table 1~~)~~.

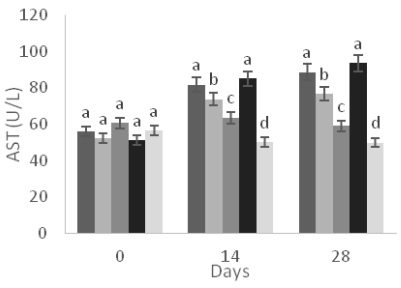
Table 1. Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %

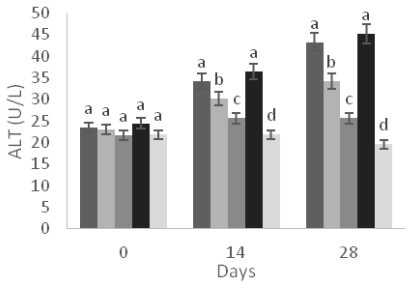
Phytochemical screening showed that 70% ethanol extract of *P. scutellarioides* leaves contained flavonoids, phenols, saponins, and triterpenoids. The results showed that the total flavonoid level of 70% ethanol extract of *P. scutellarioides* leaves was 12.62 mg QE/g extract.

Hepatoprotective~~or~~ Activity of P. scutellarioides leaves extract

This study used 50mg/kg of ~~used a high dose of anti-TB drugs,~~ isoniazid, and rifampicin to induce liver damage in rats. *P. scutellarioides* leave extract was given concurrently with the drugs for 28 days. The hepatoprotective activities were examined by measuring the levels of serum total bilirubin, direct bilirubin, AST, ALT, albumin, liver lipid peroxidation, and histology examination (Figs 1 A – E).



A



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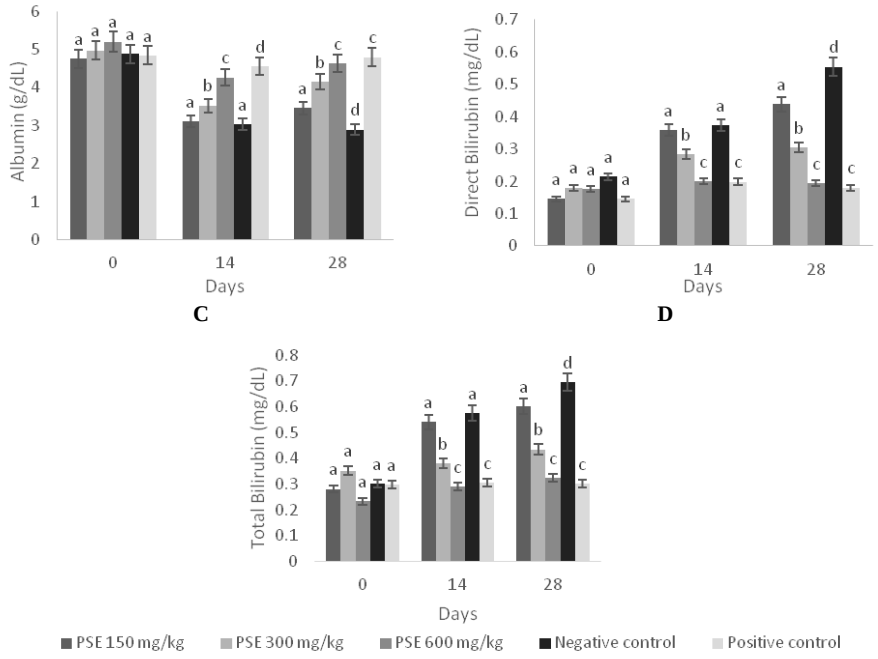

Figure 1. Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A)aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different (p<0,05)

Isoniazid and rifampicin ~~As seen in Figure 1, the TB drugs~~ caused liver injury, as indicated by the drastic change in all hepatic biochemical parameters since day 14. On the other hand, PSE groups showed improvement in all parameters, except for 150mg/kg~~the lowest dose~~. The results~~data~~ show~~ed~~ that PSE treatment groups showed a significant difference in total bilirubin levels, direct bilirubin, and albumin ($p < 0.05$) against negative controls at 300 and 600 mg/kg doses. The same trend was seen in the ALT and AST levels. It was alsoobserved ~~is also noticeable~~ that 600mg/kg~~the highest dose~~ of PSE ~~could~~ result~~ed~~ in stable hepatic parameters and almost no chance compared to day 0 (Fig2 A - C~~before treatment~~).

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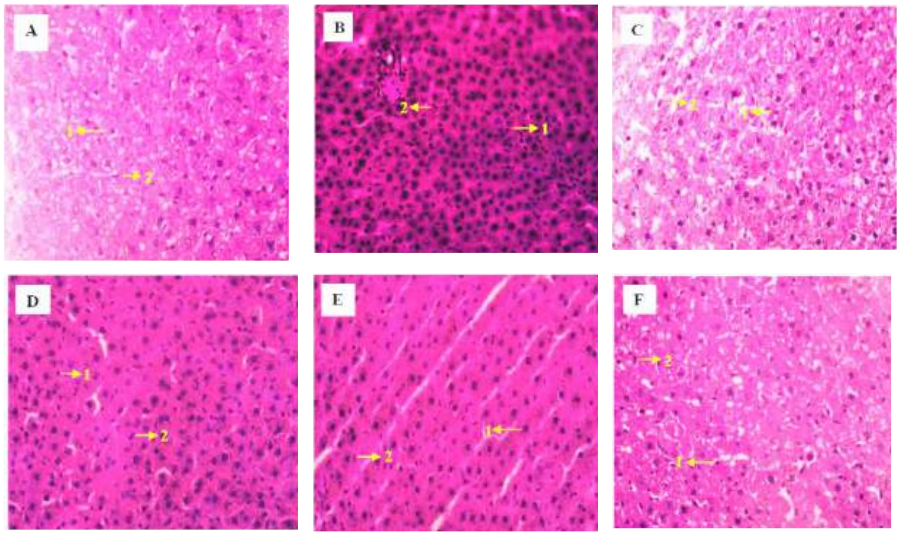

Figure 2. Histological Observation ~~Results~~ of Livers ~~Organs~~ (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150mg/kg (E) PSE 300mg/kg (F) PSE 600mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2. The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 [d]	75.32 ± 6.21 [d]	19.51±0.42 [a]
PSE 300 mg/kg	93.00 ± 4.69 [e]	58.45 ± 7.84 [e]	17.70±0.45 [b]
PSE 600 mg/kg	71.55 ± 11.1 [c]	41.25 ± 3.10 [c]	15.63±0.69 [d]
Legalon®	62.80 ± 8.03 [c]	31.70 ± 2.70 [c]	13.04±0.83 [c]
Negative control	136.2 ± 7.99 [b]	88.00 ± 7.25 [b]	21.90±0.84 [e]
Normal control	47.90 ± 7.95[a]	21.80 ± 3.29 [a]	12.74±1.07 [c]

The same subscript in each column shows comparable results (p>0.05), PSE (*Plectranthus scutellarioides* Leaves extract)

Histological observations of liver cells show that isoniazid and rifampicin cause inflammation and cell death (necrosis), which is in line with other studies, where these TB drugs induce inflammation characterized by an increase in the number of Kupffer cells. The drugs also cause cell necrosis, as indicated by a shrinking and blackish cell nucleus (pyknotic nucleus) [11]. PSE co-administration with isoniazid and rifampicin ~~the TB drugs~~ showed a noticeable histology difference compared to the negative control, especially at 600 mg/kg. PSE could reduce damage in the liver shown by Kupffer cells and the pyknotic nuclei in liver cells (Table 3). Figure 2 depicts a similar observation in Legalon® and PSE 600 mg/kg with normal control, suggesting the ~~decent~~ hepatoprotective effect of these groups.

Commented [CFP8]: Refereences

Discussion

Drug-induced liver injury (DILI) has been reported to occur in 19 per 10.000 people a year [12]. DILI can occur through an oxidative stress cascade, where the drug or its metabolites increase the reactive oxygen species (ROS), damaging the mitochondria and triggering immune reactions, thus initiating apoptosis or necrosis and causing cell death [13]. This event eventually causes acute or chronic liver disease [14]. The present study focuses on the approach to prevent liver injury against rifampicin and isoniazid toxicity. Both ~~of these~~ drugs have been known to cause hepatotoxicity in tuberculosis patients. It causes membrane damage, which increases liver enzymes and bilirubin in the systemic circulation, causing an imbalance in endogenous antioxidants and increased lipid peroxidation [15].
The liver enzyme levels in the systemic circulation are indicative of ~~ed~~ liver cell damage, whereas bilirubin and albumin levels are associated with liver function. Measurement of bilirubin levels in this study was done by the Jendrassik-Grof method, where the bilirubin reacts with Diazotized Sulfanilic Acid (DSA) and forms azobilirubin. Total bilirubin consists of direct (conjugated) and indirect (unconjugated) bilirubin, where glucuronic acid, with the help of uridine diphosphate, conjugates with the bilirubin to form direct bilirubin [16].

The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the ~~TB~~ drugs ~~results in~~ significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result ~~can be~~ seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST[17]. ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels[18]. PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum*[19]. It could prevent damage caused by tuberculosis drugs through a mechanism of reducing oxidative stress and lipid peroxidation[20]. Silymarin stabilizes the reactive oxygen species

(ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders[21].

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory response[11]. Kupffer cells have an oval shape on the surface of cells in the sinusoid[22]. The identical observation was also seen in humans. Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils[23]. The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids. The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 3).

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus*, *P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats[24]. *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg [25] and *Alpinia Officinarum at* a dose of 400 mg/kg[26], showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of interest:

None declared.

Acknowledgments:

This work was supported by Lembaga Penelitian dan Pengembangan Universitas Muhammadiyah Prof DR HAMKA

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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Thu, Aug 25, 2022 at 1:34 PM
To: Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com>

Dear Prof. Abiodun

We attach comments and corrected scripts. Thanks.

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Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com> Thu, Aug 25, 2022 at 2:16 PM
To: Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id>

Thanks for the revised manuscript

Best regards

Abiodun

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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Sun, Sep 25, 2022 at 4:24 PM
To: Editor-in-Chief Tjnpr <editor.tjnpr@gmail.com>

Dear Prof Abiodun,

We would like to ask about our paper entitled: "Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats" which information from the editor will be published in Vol 6 issue 8. However, after we checked the TJNPR website in the current issue section does not appear our journal release. Even though we have paid the publication fee and made revisions to the manuscript according to the reviewer's input. Is there a problem with our paper that hasn't been published yet? Please give us an explanation. Thanks.

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Thank you. The manuscript will be published in the upcoming issue vol 6, issue 9.

Best regards

Abiodun

Professor Abiodun Falodun, PhD
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Editorial Team <et.tjnpr@gmail.com> Wed, Oct 5, 2022 at 4:39 PM
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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Thu, Oct 6, 2022 at 10:15 AM
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For the attached manuscript with the title "***Plectranthus scutellarioides* Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats**" to be processed for galley proof, kindly respond to the following comments;

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2. Include the date (month and year) of plant leaves collection and the voucher number of the material
3. See the comments made in the Reference section.

Conflict of interest session should be included, and if there is no conflict of interest, this should be stated clearly as follows; **The authors declare no conflict of interest**.

A declaration of the liability of the authors for claims relating to the content of this article should also be included when submitting the revised manuscript. This should be stated as follows;

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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Fri, Oct 7, 2022 at 7:41 AM
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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Fri, Dec 2, 2022 at 8:37 AM
To: Editorial Team <et.tjnpr@gmail.com>

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We have a question regarding an article entitled: "Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats"

Are there still parts of the manuscript that we need to fix again? we request the information immediately.

Regards, Ermi

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Ni Putu Ermi Hikmawanti <ermy0907@uhamka.ac.id> Fri, Dec 23, 2022 at 2:41 PM
To: Editorial Team <et.tjnpr@gmail.com>

Dear Editors,

We have a question regarding an article entitled: "Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats"

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Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

ABSTRACT

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential as a heaprotector. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

Keywords: Liver injury, *Plectranthus scutellarioides*, antioxidant

Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver.[1] A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity.[2] The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage.[3] Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipta alba*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Mimosa pudica*, and *Silybum marianum*,[4] where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk,[5] as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities.[6,7] The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-O-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-O-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity.[8] This study evaluated whether *P. scutellarioides* could reduce liver damage by isoniazid and rifampicin .

Materials and methods

P. scutellarioides leaves Extraction

The leaves were obtained from Balitro Indonesia, on February 2019, and identified and authenticated as *Plectranthus scutellarioides (L.) R. Br.* from the family *Lamiaceae*. A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated for 24 hours. The maceration was done seven times with new solvent. The filtrate was evaporated in a vacuum rotary evaporator (50°C) and then continued with a water bath (50°C) to obtain the thick extract.

Determination of P. scutellarioides Leaves Extract Water Content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of Loss Drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Alkaloid Identification

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0.5 g thick extract, added with 8 ml of 2N HCl, then heated, cooled, and strained. The filtrate was divided into four parts: the first tube was blank, and the second part was added with Bouchardat reagent, where blackish-brown deposits indicate the presence of alkaloids. In the third tube, the Dragendorff reagent was added, where the formation of brown-orange precipitate shows the presence of alkaloids, and in tube 4, a Mayer reagent was added, where a yellowish-white precipitate indicates the presence of an alkaloid (Ministry of Health Republic of Indonesia, 2008).

Flavonoid Identification

0.5 g thick extract was added with 2 mL of ethanol, heated then filtered. Then ten drops of concentrated HCl and 0.1 g of Mg metal were added to the filtrate. The red color indicates the presence of flavonoids.

Phenolic Identification

0.5 g of thick extract was put into a test tube and then added with 2 mL of ethanol, then 2-3 drops of 5% $FeCl_3$. The formation of a color change from the initial color of light green to black-blue indicates the presence of phenolic.

Saponin Identification

0.5 g of thick extract plus 10 mL of hot water, cooled and shaken vigorously for 30 seconds. The presence of saponin was indicated by 1-10 cm froth formation, which lasted for 10 seconds, and the foam did not disappear after being added with one drop of HCl 2N.

Tannin Identification

0.5 g of thick extract was added with 15 mL of water, homogenized, transferred into a test tube, boiled for several minutes, and then filtered. The filtrate was added with 10% gelatine and the formation of white deposits indicates the presence of tannin.

Identification of Triterpenoids and Steroids

0.5 g of thick extract was added with 5 mL of ethanol, heated, then filtered and cooled. The filtrate was evaporated and then added with three drops of ether, three drops of anhydrous acetic acid, and one drop of concentrated sulfuric acid. The formation of the red color indicates the presence of triterpenoids, and the formation of the green color indicates the presence of steroids.

Determination of Total Flavonoid Levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

$$Total\ Flavonoid = \frac{Flavonoid\ consentration\ (\mu g/mL) \times sample\ volume\ (mL) \times dilution\ factor}{Extract\ weigh}$$

Animals and experimental design

Wistar rats (200-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25° C and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control and positive control (Silymarin) 100 mg/kg . All administration was done orally, 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg p.o orally every day for 28 days. The next day, the rats were sacrificed by cervical dislocation.

Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells,[10] were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with 1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloracetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbiturate (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results

Plectranthus scutellarioides leaves extract characteristics

Ethanol extract of *P. scutellarioides* leaves was obtained in the form of concentrated blackish-brown extract and a slightly bitter taste (Table 1).

Phytochemical screening showed that 70% ethanol extract of *P. scutellarioides* leaves contained flavonoids, phenols, saponins, and triterpenoids. The results showed that the total flavonoid level of 70% ethanol extract of *P. scutellarioides* leaves was 12.62 mg QE/g extract.

Hepatoprotective Activity of P. scutellarioides leaves extract

This study used 50mg/kg of isoniazid, and rifampicin to induce liver damage in rats. *P. scutellarioides* leave extract was given concurrently with the drugs for 28 days. The hepatoprotective activities were examined by measuring the levels of serum total bilirubin, direct bilirubin, AST, ALT, albumin, liver lipid peroxidation, and histology examination (Figs 1 A – E).

Isoniazid and rifampicin caused liver injury, as indicated by the drastic change in all hepatic biochemical parameters since day 14. On the other hand, PSE groups showed improvement in all parameters, except for 150mg/kg. The results show that PSE treatment groups showed a significant decreased in total bilirubin levels and direct bilirubin, while the albumin level was higher ($p < 0.05$) then negative controls at 300 and 600 mg/kg doses. The same trend was seen in the ALT and AST levels. It was also observed that 600mg/kg of PSE resulted in stable hepatic parameters and almost no chance compared to day 0 (Fig2 A - C).

Histological observations of liver cells show that isoniazid and rifampicin cause inflammation and cell death (necrosis), which is in line with other studies(11), where these TB drugs induce inflammation characterized by an increase in the number of Kupffer cells. The drugs also cause cell necrosis, as indicated by a shrinking and blackish cell nucleus (pyknotic nucleus).[11] PSE co-administration with isoniazid and rifampicin showed a noticeable histology difference compared to the negative control, especially at 600 mg/kg. PSE could reduce damage in the liver shown by Kupffer cells and the pyknotic nuclei in liver cells (Table 3). Figure 2 depicts a similar observation in Legalon® and PSE 600 mg/kg with normal control, suggesting the hepatoprotective effect of these groups.

Discussion

Drug-induced liver injury (DILI) has been reported to occur in 19 per 10.000 people a year.[12] DILI can occur through an oxidative stress cascade, where the drug or its metabolites increase the reactive oxygen species (ROS), damaging the mitochondria and triggering immune reactions, thus initiating apoptosis or necrosis and causing cell death.[13] This event eventually causes acute or chronic liver disease.[14] The present study focuses on the approach to prevent liver injury against rifampicin and isoniazid toxicity. Both drugs have been known to cause hepatotoxicity in tuberculosis patients. It causes membrane

damage, which increases liver enzymes and bilirubin in the systemic circulation, causing an imbalance in endogenous antioxidants and increased lipid peroxidation.[15]
The liver enzyme levels in the systemic circulation are indicative of liver cell damage, whereas bilirubin and albumin levels are associated with liver function. Measurement of bilirubin levels in this study was done by the Jendrassik-Grof method, where the bilirubin reacts with Diazotized Sulfanilic Acid (DSA) and forms azobilirubin. Total bilirubin consists of direct (conjugated) and indirect (unconjugated) bilirubin, where glucuronic acid, with the help of uridine diphosphate, conjugates with the bilirubin to form direct bilirubin.[16]

The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the drugs significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST.[17] ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels.[18] PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum.*[19] It could prevent damage caused by tuberculosis drugs through a mechanism of reducing oxidative stress and lipid peroxidation.[20] Silymarin stabilizes the reactive oxygen species (ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders.[21]

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory

response(11). Kupffer cells have an oval shape on the surface of cells in the sinusoid.[22] The identical observation was also seen in humans. Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils.[23] The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids. The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 3).

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus, P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats.[24] *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg[25] and *Alpinia Officinarum at* a dose of 400 mg/kg,[26] showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of interest:
None declared.

Acknowledgments:
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Abbreviate all Journal names

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Table 1. Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %

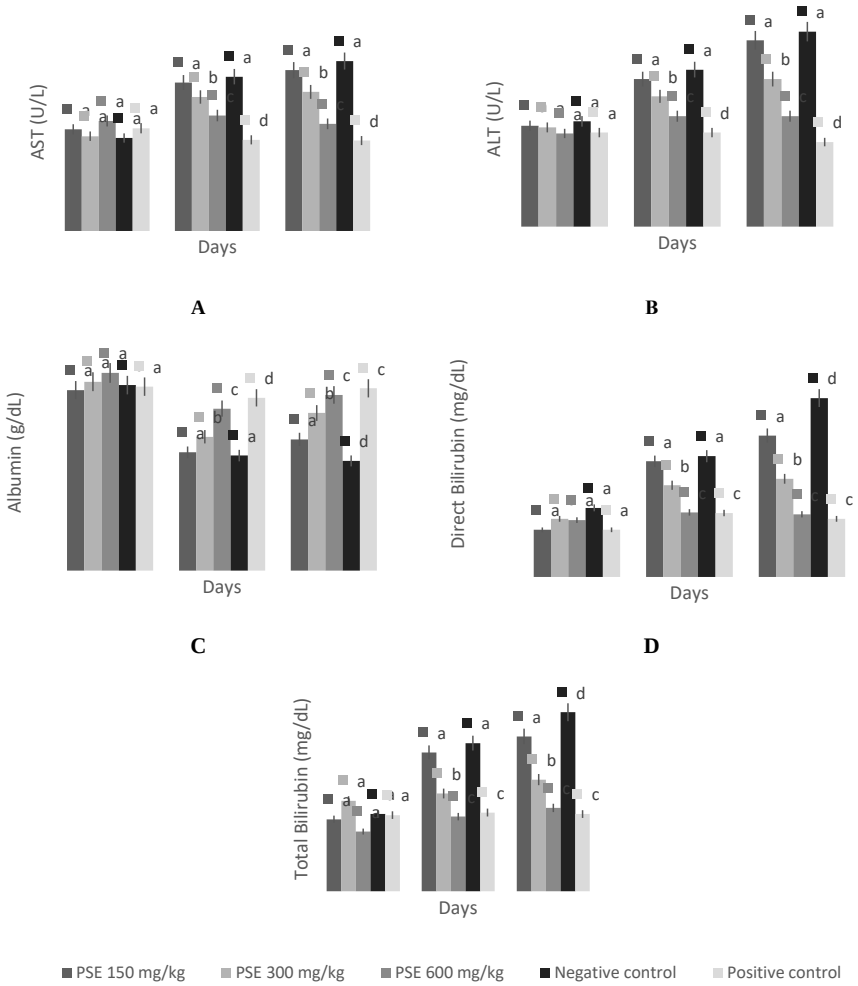

Figure 1. Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A)aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different ($p<0,05$)

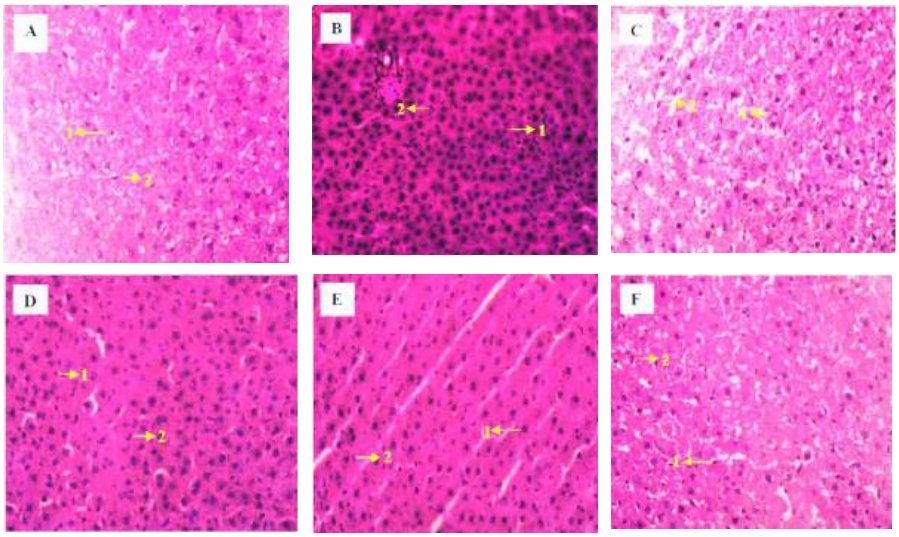

Figure 2. Histological Observation of Livers (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150mg/kg (E) PSE 300mg/kg (F) PSE 600mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2. The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 ^{d}	75.32 ± 6.21 ^{d}	19.51±0.42 ^{a}
PSE 300 mg/kg	93.00 ± 4.69 ^{e}	58.45 ± 7.84 ^{e}	17.70±0.45 ^{b}
PSE 600 mg/kg	71.55 ± 11.1 ^{c}	41.25 ± 3.10 ^{c}	15.63±0.69 ^{d}
Legalon®	62.80 ± 8.03 ^{c}	31.70 ± 2.70 ^{c}	13.04±0.83 ^{c}
Negative control	136.2 ± 7.99 ^{b}	88.00 ± 7.25 ^{b}	21.90±0.84 ^{e}
Normal control	47.90 ± 7.95^{a}	21.80 ± 3.29 ^{a}	12.74±1.07 ^{c}

The same subscript in each column shows comparable results (p>0.05), PSE (*Plectranthus scutellarioides* Leaves extract)

Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

ABSTRACT

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential as a heaprotector. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

Keywords: Liver injury, *Plectranthus scutellarioides*, antioxidant

Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver (1). A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity (2). The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage (3). Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipta alba*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Mimosa pudica*, and *Silybum marianum* (4), where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk (5), as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities (6,7). The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-O-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-O-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity (8). This study evaluated whether *P. scutellarioides* could reduce liver damage by isoniazid and rifampicin .

Materials and methods

P. scutellarioides leaves Extraction

The leaves were obtained from Balitro, Indonesia and identified and authenticated as *Plectranthus scutellarioides (L.) R. Br.* from the family *Lamiaceae.* A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated for 24 hours. The maceration was done seven times with new solvent. The filtrate was evaporated in a vacuum rotary evaporator (50°C) and then continued with a water bath (50°C) to obtain the thick extract.

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Determination of P. scutellarioides Leaves Extract Water Content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of Loss Drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Alkaloid Identification

0.5 g thick extract, added with 8 ml of 2N HCl, then heated, cooled, and strained. The filtrate was divided into four parts: the first tube was blank, and the second part was added with Bouchardat reagent, where blackish-brown deposits indicate the presence of alkaloids. In the third tube, the Dragendorff reagent was added, where the formation of brown-orange precipitate shows the presence of alkaloids, and in tube 4, a Mayer reagent was added, where a yellowish-white precipitate indicates the presence of an alkaloid (Ministry of Health Republic of Indonesia, 2008).

Flavonoid Identification

0.5 g thick extract was added with 2 mL of ethanol, heated then filtered. Then ten drops of concentrated HCl and 0.1 g of Mg metal were added to the filtrate. The red color indicates the presence of flavonoids.

Phenolic Identification

0.5 g of thick extract was put into a test tube and then added with 2 mL of ethanol, then 2-3 drops of 5% $FeCl_3$. The formation of a color change from the initial color of light green to black-blue indicates the presence of phenolic.

Saponin Identification

0.5 g of thick extract plus 10 mL of hot water, cooled and shaken vigorously for 30 seconds. The presence of saponin was indicated by 1-10 cm froth formation, which lasted for 10 seconds, and the foam did not disappear after being added with one drop of HCl 2N.

Tannin Identification

0.5 g of thick extract was added with 15 mL of water, homogenized, transferred into a test tube, boiled for several minutes, and then filtered. The filtrate was added with 10% gelatine and the formation of white deposits indicates the presence of tannin.

Identification of Triterpenoids and Steroids

0.5 g of thick extract was added with 5 mL of ethanol, heated, then filtered and cooled. The filtrate was evaporated and then added with three drops of ether, three drops of anhydrous acetic acid, and one drop of concentrated sulfuric acid. The formation of the red color indicates the presence of triterpenoids, and the formation of the green color indicates the presence of steroids.

Determination of Total Flavonoid Levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

$$Total\ Flavonoid = \frac{Flavonoid\ consentration\ (\mu g/mL) \times sample\ volume\ (mL) \times dilution\ factor}{Extract\ weigh}$$

Animals and experimental design

Wistar rats (200-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25° C and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control and positive control (Silymarin) 100 mg/kg . All administration was done orally, 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg p.o orally every day for 28 days. The next day, the rats were sacrificed by cervical dislocation.

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Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells (10), were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with 1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloracetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbiturate (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results

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The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the drugs significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST(17). ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels(18). PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum*(19). It could prevent damage caused by tuberculosis drugs through a mechanism of reducing oxidative stress and lipid peroxidation(20). Silymarin stabilizes the reactive oxygen species (ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders(21).

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory response(11). Kupffer cells have an oval shape on the surface of cells in the sinusoid(22). The identical observation was also seen in humans. Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils(23). The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids. The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 3).

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus*, *P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats(24). *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg (25) and *Alpinia Officinarum at* a dose of 400 mg/kg(26), showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of interest:

None declared.

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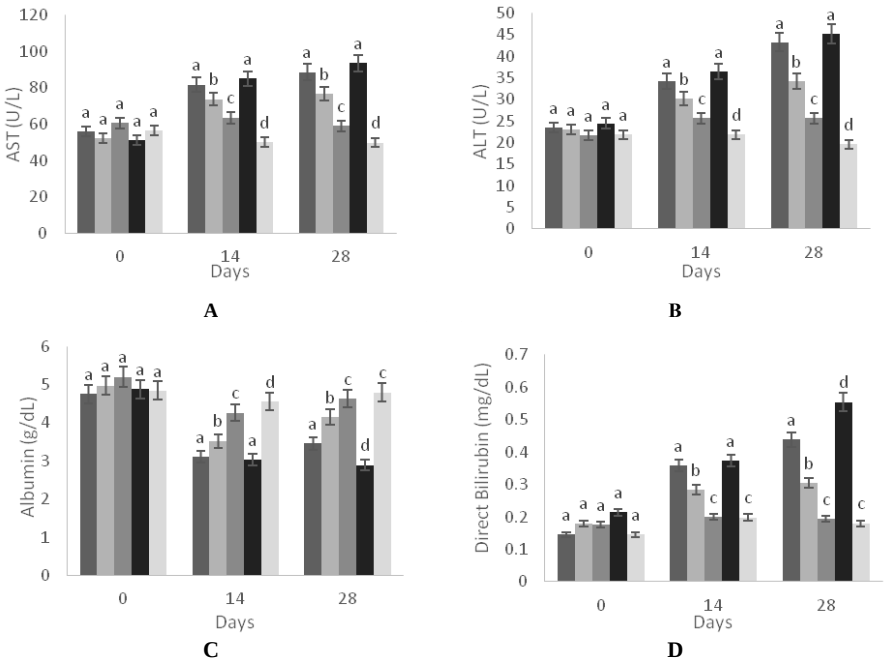
Table 1. Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %



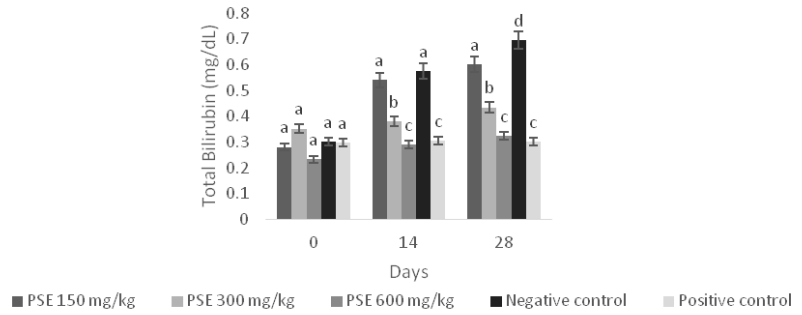


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Figure 1. Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A)aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different ($p<0,05$)

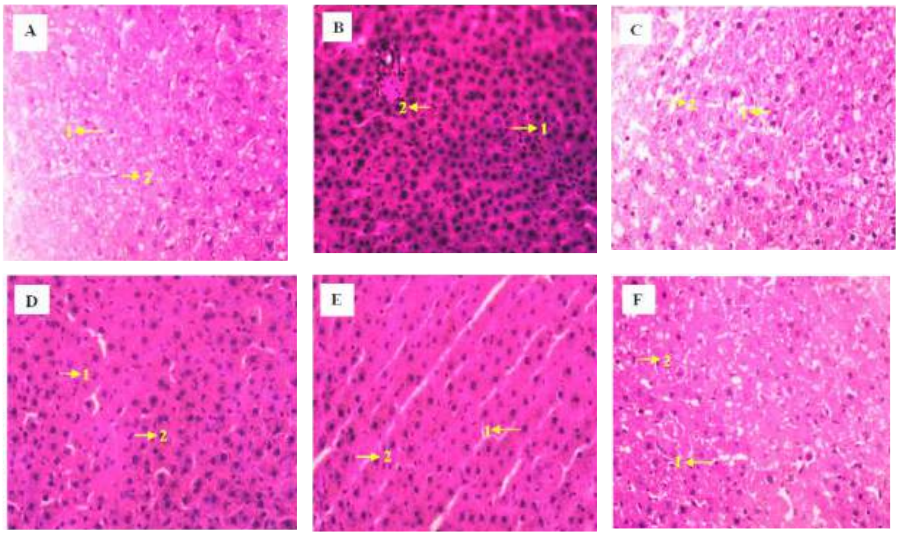

Figure 2. Histological Observation of Livers (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150mg/kg (E) PSE 300mg/kg (F) PSE 600mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2. The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 ^{d}	75.32 ± 6.21 ^{d}	19.51±0.42 ^{a}
PSE 300 mg/kg	93.00 ± 4.69 ^{e}	58.45 ± 7.84 ^{e}	17.70±0.45 ^{b}
PSE 600 mg/kg	71.55 ± 11.1 ^{c}	41.25 ± 3.10 ^{c}	15.63±0.69 ^{d}
Legalon®	62.80 ± 8.03 ^{c}	31.70 ± 2.70 ^{c}	13.04±0.83 ^{c}
Negative control	136.2 ± 7.99 ^{b}	88.00 ± 7.25 ^{b}	21.90±0.84 ^{e}
Normal control	47.90 ± 7.95^{a}	21.80 ± 3.29 ^{a}	12.74±1.07 ^{c}

The same subscript in each column shows comparable results ($p>0.05$), PSE (*Plectranthus scutellarioides* Leaves extract)

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Original Research Article

Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

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ABSTRACT

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential with hepatoprotection effect. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

Keywords: Antioxidant, Flavonoids, Lamiaceae, Liver injury, Hepatoprotection, *Plectranthus scutellarioides* .

Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver.[1] A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity.[2] The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage.[3] Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipta alba*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Mimosa pudica*, and *Silybum marianum*,[4] where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk,[5] as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities.[6,7] The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-O-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-O-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity.[8] This study evaluated whether *P. scutellarioides* could reduce liver damage by isoniazid and rifampicin.

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Materials and methods

P. scutellarioides leaves extraction

The leaves were obtained from Balitro, Indonesia and identified and authenticated as *Plectranthus scutellarioides* (L.) R. Br. from the family *Lamiaceae.* A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated for 24 hours. The maceration was done seven times with new solvent. The filtrate was evaporated in a vacuum rotary evaporator (50°C) and then continued with a water bath (50°C) to obtain the thick extract.

Determination of P. scutellarioides leaves extract water content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of loss drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Phytochemical identification

The qualitatively identification of phytochemical from *P. scutellarioides Leaves Extract* was conducted following the procedure in Indonesian Herbal Pharmacopeia.[9]

Determination of total flavonoid levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

$$\text{Total Flavonoid} = \frac{\text{Flavonoid consentration } (\mu g/mL) \times \text{sample volume } (mL) \times \text{dilution factor}}{\text{Extract weigh}}$$

Animals and experimental design

Wistar rats (200-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25 °C and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control and positive control (Silymarin) 100 mg/kg. All administration was done orally, 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg po. orally every day for 28 days. The next day, the rats were sacrificed by cervical dislocation.

Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells,[10] were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with 1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloroacetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbiturate (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results and Discussion

Plectranthus scutellarioides leaves extract characteristics

Ethanol extract of *P. scutellarioides* leaves was obtained in the form of concentrated blackish-brown extract and a slightly bitter taste (Table 1). Phytochemical screening showed that 70% ethanol extract of *P. scutellarioides* leaves contained flavonoids, phenols, saponins, and triterpenoids. The results showed that the total flavonoid level of 70% ethanol extract of *P. scutellarioides* leaves was 12.62 mg QE/g extract. Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids.

Table 1: Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %

The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 3).

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Hepatoprotective Activity of P. scutellarioides leaves extract

Drug-induced liver injury (DILI) has been reported to occur in 19 per 10.000 people a year.[12] DILI can occur through an oxidative stress cascade, where the drug or its metabolites increase the reactive oxygen species (ROS), damaging the mitochondria and triggering immune reactions, thus initiating apoptosis or necrosis and causing cell death.[13] This event eventually causes acute or chronic liver disease.[14] The present study focuses on the approach to prevent liver injury against rifampicin and isoniazid toxicity. Both drugs have been known to cause hepatotoxicity in tuberculosis patients. It causes membrane damage, which increases liver enzymes and bilirubin in the systemic circulation, causing an imbalance in endogenous antioxidants and increased lipid peroxidation.[15]

This study used 50 mg/kg of isoniazid, and rifampicin to induce liver damage in rats. *P. scutellarioides* leave extract was given concurrently with the drugs for 28 days. The hepatoprotective activities were examined by measuring the levels of serum total bilirubin, direct bilirubin, AST, ALT, albumin, liver lipid peroxidation, and histology examination (Figure 1 A – E).

The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the drugs significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Isoniazid and rifampicin caused liver injury, as indicated by the drastic change in all hepatic biochemical parameters since day 14. On the other hand, PSE groups showed improvement in all parameters, except for 150 mg/kg. The results show that PSE treatment groups showed a significant decreased in total bilirubin levels and direct bilirubin, while the albumin level was higher ($p < 0.05$) then negative controls at 300 and 600 mg/kg doses. The same trend was seen in the ALT and AST levels. It was also observed that 600 mg/kg of PSE resulted in stable hepatic parameters and almost no chance compared to day 0 (Figure 2A - C).

The liver enzyme levels in the systemic circulation are indicative of liver cell damage, whereas bilirubin and albumin levels are associated with liver function. Measurement of bilirubin levels in this study was done by the Jendrassik-Grof method, where the bilirubin reacts with Diazotized Sulfanilic Acid (DSA) and forms azobilirubin. Total bilirubin consists of direct (conjugated) and indirect (unconjugated) bilirubin, where glucuronic acid, with the help of uridine diphosphate, conjugates with the bilirubin to form direct bilirubin.[16]

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Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST.[17] ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels.[18] PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum.*[19] It could prevent damage caused by tuberculosis drugs through a mechanism of reducing oxidative stress

and lipid peroxidation.[20] Silymarin stabilizes the reactive oxygen species (ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders.[21]

Histological observations of liver cells show that isoniazid and rifampicin cause inflammation and cell death (necrosis), which is in line with other studies,[11] where these TB drugs induce inflammation characterized by an increase in the number of Kupffer cells. The drugs also cause cell necrosis, as indicated by a shrinking and blackish cell nucleus (pyknotic nucleus).[11] PSE co-administration with isoniazid and rifampicin showed a noticeable histology difference compared to the negative control, especially at 600 mg/kg. PSE could reduce damage in the liver shown by Kupffer cells and the pyknotic nuclei in liver cells (Table 3). Figure 2 depicts a similar observation in Legalon® and PSE 600 mg/kg with normal control, suggesting the hepatoprotective effect of these groups.

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory response.[11] Kupffer cells have an oval shape on the surface of cells in the sinusoid.[22] The identical observation was also seen in humans.

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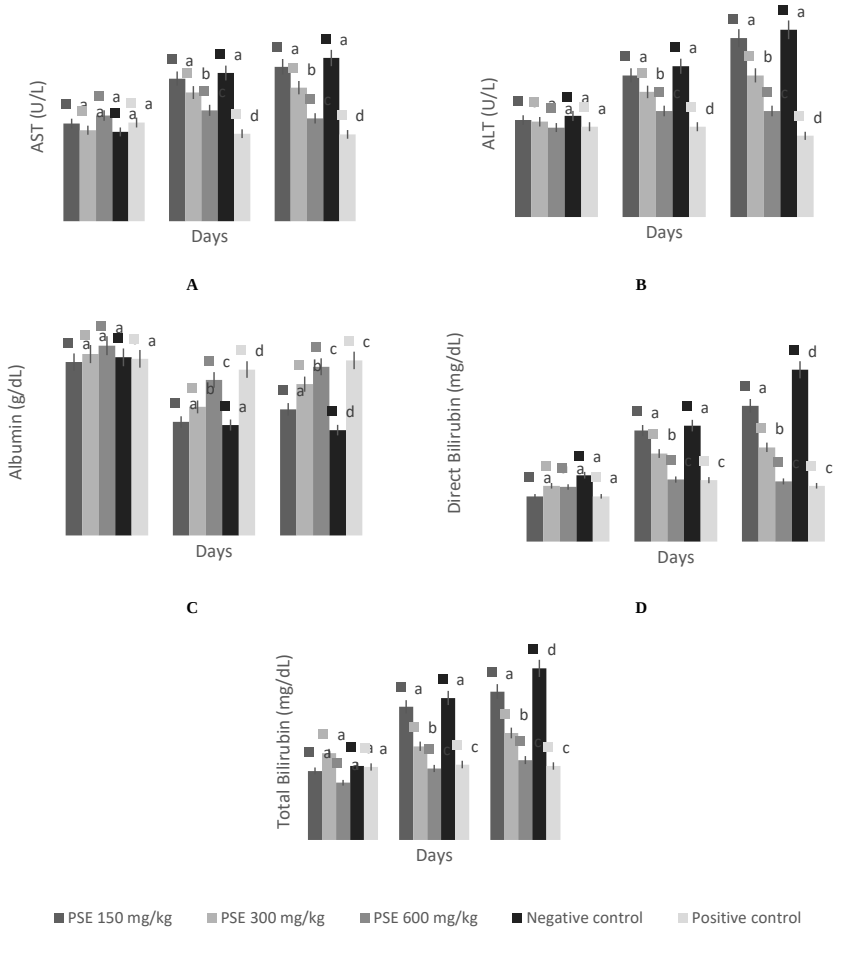

Figure 1: Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A)aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different (p<0,05)

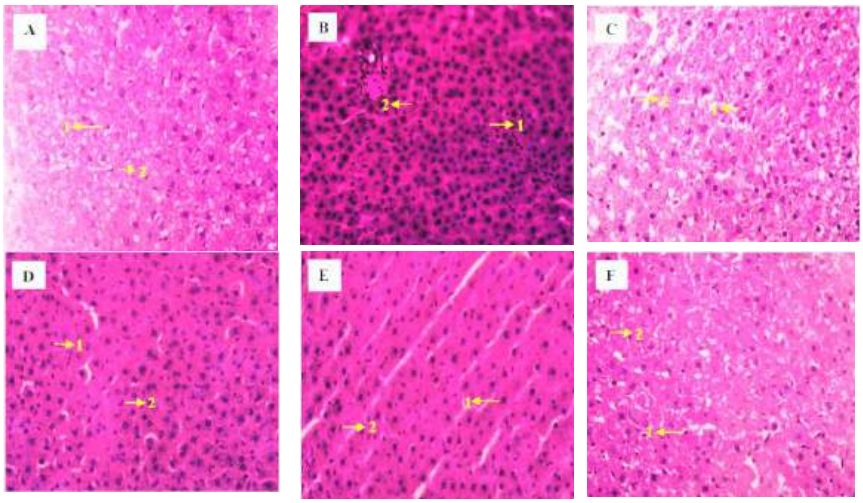

Figure 2: Histological Observation of Livers (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150 mg/kg (E) PSE 300 mg/kg (F) PSE 600 mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2: The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 [d]	75.32 ± 6.21 [d]	19.51 ± 0.42
PSE 300 mg/kg	93.00 ± 4.69 [e]	58.45 ± 7.84 [e]	17.70 ± 0.45 [b]
PSE 600 mg/kg	71.55 ± 11.1 [c]	41.25 ± 3.10 [c]	15.63 ± 0.69 [d]
Legalon®	62.80 ± 8.03 [c]	31.70 ± 2.70	13.04 ± 0.83
Negative control	136.2 ± 7.99 [b]	88.00 ± 7.25	21.90 ± 0.84 [e]
Normal control	47.90 ± 7.95[a]	21.80 ± 3.29 [a]	12.74 ± 1.07 [c]

The same subscript in each column shows comparable results (p>0.05), PSE (*Plectranthus scutellarioides* Leaves extract)

Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils.[23] The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus*, *P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats.[24] *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg[25] and *Alpinia Officinarum at* a dose of 400 mg/kg,[26] showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Original Research Article

Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

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ABSTRACT

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential with hepatoprotection effect. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

***Keywords*:** Antioxidant, Flavonoids, Lamiaceae, Liver injury, Hepatoprotection, *Plectranthus scutellarioides* .

Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver.[1] A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity.[2] The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage.[3] Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipta alba*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Mimosa pudica*, and *Silybum marianum*,[4] where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk,[5] as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities.[6,7] The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-O-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-O-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity.[8] This study evaluated whether *P. scutellarioides* could reduce liver damage by isoniazid and rifampicin.

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Materials and methods

P. scutellarioides leaves extraction

The leaves were obtained from Balitro, Indonesia and identified and authenticated as *Plectranthus scutellarioides* (L.) R. Br. from the family *Lamiaceae*. A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated for 24 hours. The maceration was done seven times with new solvent. The filtrate was evaporated in a vacuum rotary evaporator (50°C) and then continued with a water bath (50°C) to obtain the thick extract.

Determination of P. scutellarioides leaves extract water content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of loss drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Phytochemical identification

The qualitatively identification of phytochemical from *P. scutellarioides Leaves Extract* was conducted following the procedure in Indonesian Herbal Pharmacopeia.[9]

Determination of total flavonoid levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

$$\text{Total Flavonoid} = \frac{\text{Flavonoid consentration } (\mu g/mL) \times \text{sample volume } (mL) \times \text{dilution factor}}{\text{Extract weigh } (g)}$$

Animals and experimental design

Wistar rats (200-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25 °C and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control and positive control (Silymarin) 100 mg/kg. All administration was done orally, 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg po. orally every day for 28 days. The next day, the rats were sacrificed by cervical dislocation.

Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells,[10] were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with 1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloroacetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbiturate (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results and Discussion

Plectranthus scutellarioides leaves extract characteristics

Ethanol extract of *P. scutellarioides* leaves was obtained in the form of concentrated blackish-brown extract and a slightly bitter taste. The characteristics of *P. scutellarioides* leaves extract are presented in Table 1. Phytochemical screening showed that 70% ethanol extract of *P. scutellarioides* leaves contained flavonoids, phenols, saponins, and triterpenoids. The results showed that the total flavonoid level of 70% ethanol extract of *P. scutellarioides* leaves was 12.62 mg QE/g extract. Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids.

Table 1: Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %

The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 2).

Hepatoprotective Activity of P. scutellarioides leaves extract

Drug-induced liver injury (DILI) has been reported to occur in 19 per 10.000 people a year.[12] DILI can occur through an oxidative stress cascade, where the drug or its metabolites increase the reactive oxygen species (ROS), damaging the mitochondria and triggering immune reactions, thus initiating apoptosis or necrosis and causing cell death.[13] This event eventually causes acute or chronic liver disease.[14] The present study focuses on the approach to prevent liver injury against rifampicin and isoniazid toxicity. Both drugs have been known to cause hepatotoxicity in tuberculosis patients. It causes membrane damage, which increases liver enzymes and bilirubin in the systemic circulation, causing an imbalance in endogenous antioxidants and increased lipid peroxidation.[15]

This study used 50 mg/kg of isoniazid, and rifampicin to induce liver damage in rats. *P. scutellarioides* leave extract was given concurrently with the drugs for 28 days. The hepatoprotective activities were examined by measuring the levels of serum total bilirubin, direct bilirubin, AST, ALT, albumin, liver lipid peroxidation, and histology examination (Figure 1 A – E).

The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the drugs significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Isoniazid and rifampicin caused liver injury, as indicated by the drastic change in all hepatic biochemical parameters since day 14. On the other hand, PSE groups showed improvement in all parameters, except for 150 mg/kg. The results show that PSE treatment groups showed a significant decreased in total bilirubin levels and direct bilirubin, while the albumin level was higher ($p < 0.05$) then negative controls at 300 and 600 mg/kg doses. The same trend was seen in the ALT and AST levels. It was also observed that 600 mg/kg of PSE resulted in stable hepatic parameters and almost no chance compared to day 0 (Figure 2A - C).

The liver enzyme levels in the systemic circulation are indicative of liver cell damage, whereas bilirubin and albumin levels are associated with liver function. Measurement of bilirubin levels in this study was done by the Jendrassik-Grof method, where the bilirubin reacts with Diazotized Sulfanilic Acid (DSA) and forms azobilirubin. Total bilirubin consists of direct (conjugated) and indirect (unconjugated) bilirubin, where glucuronic acid, with the help of uridine diphosphate, conjugates with the bilirubin to form direct bilirubin.[16]

Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST.[17] ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels.[18] PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum.*[19] It could prevent damage caused by

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tuberculosis drugs through a mechanism of reducing oxidative stress and lipid peroxidation.[20] Silymarin stabilizes the reactive oxygen species (ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders.[21]

Histological observations of liver cells show that isoniazid and rifampicin cause inflammation and cell death (necrosis), which is in line with other studies,[11] where these TB drugs induce inflammation characterized by an increase in the number of Kupffer cells. The drugs also cause cell necrosis, as indicated by a shrinking and blackish cell nucleus (pyknotic nucleus).[11] PSE co-administration with isoniazid and rifampicin showed a noticeable histology difference compared to the negative control, especially at 600 mg/kg. PSE could reduce damage in the liver shown by Kupffer cells and the pyknotic nuclei in liver cells (Table 2). Figure 2 depicts a similar observation in Legalon® and PSE 600 mg/kg with normal control, suggesting the hepatoprotective effect of these groups.

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory response.[11] Kupffer cells have an oval shape on the surface of cells in the sinusoid.[22] The identical observation was also seen in humans.

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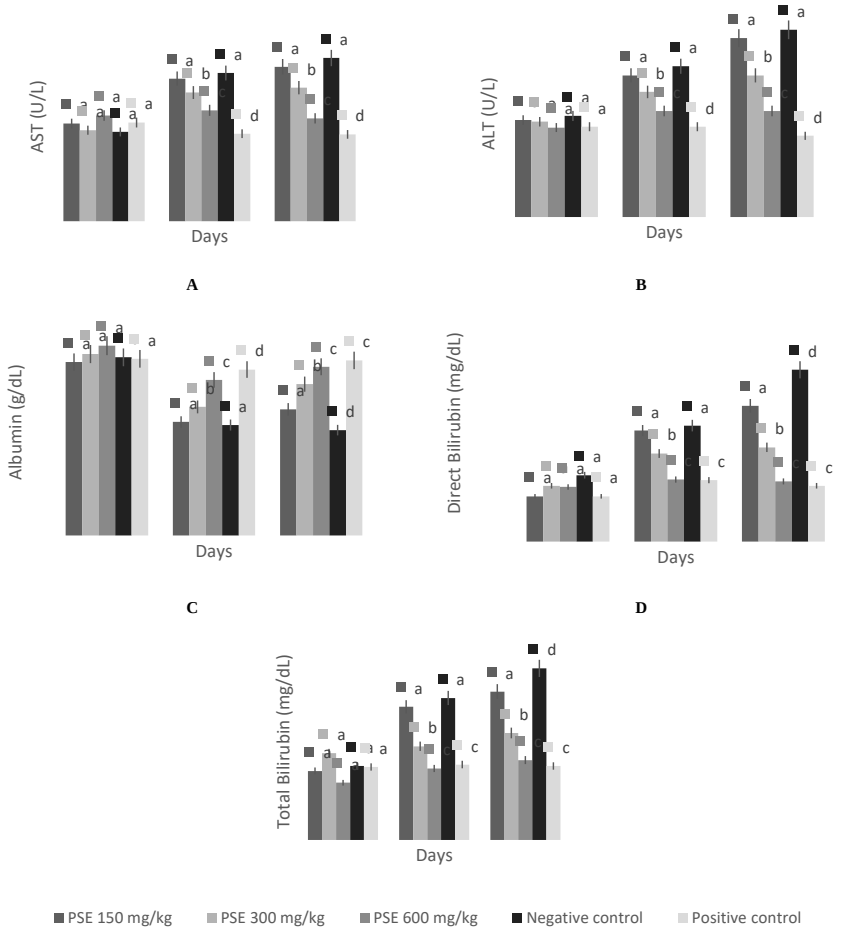

Figure 1: Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different (p<0,05)

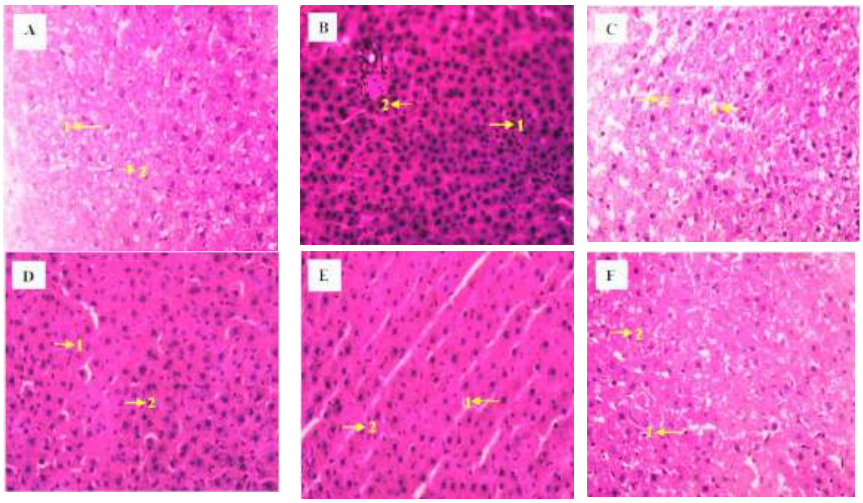

Figure 2: Histological Observation of Livers (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150 mg/kg (E) PSE 300 mg/kg (F) PSE 600 mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2: The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 [d]	75.32 ± 6.21 [d]	19.51 ± 0.42
PSE 300 mg/kg	93.00 ± 4.69 [e]	58.45 ± 7.84 [e]	17.70 ± 0.45 [b]
PSE 600 mg/kg	71.55 ± 11.1 [c]	41.25 ± 3.10 [c]	15.63 ± 0.69 [d]
Legalon®	62.80 ± 8.03 [c]	31.70 ± 2.70	13.04 ± 0.83
Negative control	136.2 ± 7.99 [b]	88.00 ± 7.25	21.90 ± 0.84 [e]
Normal control	47.90 ± 7.95[a]	21.80 ± 3.29 [a]	12.74 ± 1.07 [c]

The same subscript in each column shows comparable results (p>0.05), PSE (*Plectranthus scutellarioides* Leaves extract)

Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils.[23] The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus*, *P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats.[24] *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg[25] and *Alpinia Officinarum at* a dose of 400 mg/kg,[26] showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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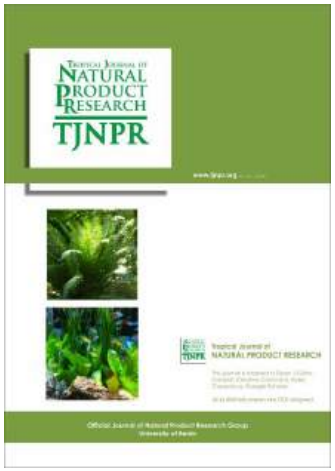

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Abstract

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential with hepatoprotection effect. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin,albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

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Original Research Article

Plectranthus scutellarioides Leaf Extract Protective Effects Against Isoniazid and Rifampicin-Induced Hepatotoxicity in Wistar Rats

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ABSTRACT

Plectranthus scutellarioides has been studied to have antioxidant and anti-inflammatory activity which shows potential with hepatoprotection effect. This study determined the hepatoprotective activity of ethanol extract of *P. scutellarioides* leaves. The rats were divided into five groups: PSE (*Plectranthus scutellarioisdes* leaves extract) 150, 300, and 600 mg/kg, negative control, and positive control (Silymarin). All groups were given the test substances concurrently with isoniazid and rifampicin (50 mg/kg) p.o every day for 28 days. The results showed that the PSE could prevent liver damage as indicated by lower levels of total bilirubin, direct bilirubin, albumin, AST, and ALT than negative controls ($p<0.005$). Histological observations of the liver confirmed these results, and this dose-dependent hepatoprotective activity was in line with the lipid peroxidation inhibition. *P. scutellarioides* extract at a dose of 600 mg/kg showed the best activity, comparable ($p> 0.05$) to Silymarin. The data suggest that the co-administration of *P. scutellarioides* leaf extract with isoniazid and rifampicin could prevent the liver injury caused by these drugs.

***Keywords*:** Antioxidant, Flavonoids, Lamiaceae, Liver injury, Hepatoprotection, *Plectranthus scutellarioides* .

Introduction

Hepatoprotector protects liver cells from damage caused by hepatotoxic substances, such as drugs, chemical compounds, and toxic substances that can damage the liver.[1] A study on tuberculosis (TB) patients showed that combination therapy of isoniazid, rifampicin, and pyrazinamide is associated with liver injury severity.[2] The metabolism of isoniazid could produce hydrazine, which is toxic to the liver. Since rifampicin increases isoniazid metabolism, concomitant use of these drugs could worsen liver damage.[3] Hepatoprotector concurrent use with TB drugs has been proven to be beneficial.

Several plants have been studied for their hepatoprotective activities, such as *Andrographis paniculata, Boerhaavia diffusa, Eclipta alba, Phyllanthus amarus, Picrorhiza kurroa, Mimosa pudica,* and *Silybum marianum*,[4] where most of these plants also showed potential antioxidant activities. Antioxidant effects have been proven beneficial in preventing antitubercular hepatotoxicity risk,[5] as well as the anti-inflammatory effect. *Plectranthus scutellarioides* leaves have shown potential for both activities.[6,7] The flavonoid compounds of *P. scutellarioides* like apigenin 7-O-(3″-*O*-acetyl)-β-D-glucuronide, apigenin 5-O-(3″-*O*-acetyl)-β-D-glucuronide and rosmarinic acid, showed potent antioxidant activity.[8] This study evaluated whether *P. scutellarioides* could reduce liver damage by isoniazid and rifampicin.

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Materials and methods

P. scutellarioides leaves extraction

The leaves were obtained from Balitro, Indonesia and identified and authenticated as *Plectranthus scutellarioides* (L.) R. Br. from the family *Lamiaceae.* A total of 680 g of leaf powder was added with 7 L of 70% ethanol and macerated for 24 hours. The maceration was done seven times with new solvent. The filtrate was evaporated in a vacuum rotary evaporator (50°C) and then continued with a water bath (50°C) to obtain the thick extract.

Determination of P. scutellarioides leaves extract water content

Determination of extract characteristics and phytochemical content screening were done following the Indonesian Herb Pharmacopoeia. Ten grams of the thick extract was weighed and added with 200 mL of water-saturated toluene. The mixture was heated for approximately 2 hours, and then the receiver tube was cooled to room temperature. The volume of water was read and then calculated in % (v/b).

Determination of loss drying

The oven was set at 105°C, and the container was heated for 30 minutes, then weighed. The extract was weighed 1 g in the container, then put in an oven at 105°C until a fixed weight was obtained.

Phytochemical identification

The qualitatively identification of phytochemical from *P. scutellarioides Leaves Extract* was conducted following the procedure in Indonesian Herbal Pharmacopeia.[9]

Determination of total flavonoid levels

Serial concentrations of each extract were added with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1 M Na-acetate, and 10 ml of distilled water. The solution was incubated for 30 minutes, and then the absorbance was read at 415 nm using Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). The flavonoid concentration was determined with quercetin as the standard.

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$$Total\ Flavonoid = \frac{Flavonoid\ consentration\ (\mu g/mL) \times sample\ volume\ (mL) \times dilution\ factor}{Extract\ weigh\ (g)}$$

Animals and experimental design

Wistar rats (200-250 grams) were used for the test. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia (404/UN2.F1/ETIK/PPM.00.02/2019). The rats were acclimatized for seven days before the experiment and kept under standard laboratory conditions (22–25 ℃ and a 12-h light/dark cycle), fed with a standard pellets diet and water ad libitum. The animals were divided into five groups: *Plectranthus scutellarioides* leaf extract (PSE) 150 mg/kg, 300 mg/kg, and 600 mg/kg, negative control and positive control (Silymarin) 100 mg/kg. All administration was done orally, 30 minutes prior to isoniazid and rifampicin. The TB drugs were given at the same dose, 50 mg/kg po. orally every day for 28 days. The next day, the rats were sacrificed by cervical dislocation.

Liver biochemical parameters

Blood samples were taken from the orbital sinuses on days 0, 14, and 28. The serum was used for total bilirubin, direct bilirubin, albumin, AST, and ALT levels. All parameters were determined using spectrophotometer clinical (Microlab 300) with a reagent supplied by HUMAN Ltd., Germany.

Histopathological studies

The livers were fixed in 10% formalin for 24 hours. Then the histology preparations were prepared with Hematoxylin-Eosin staining. Histological observations, including the liver's pyknotic nucleus and Kupffer cells,[10] were done in a 10-images view with 400× objective magnification.

Lipid peroxidation test

Lipid peroxidation of the liver was measured based on malondialdehyde (MDA) levels, with 1,1,3,3-tetra ethoxy propane (TEP) as standard. The liver was rinsed with phosphate buffer solution (PBS) pH 7.4 and dried. Then the liver was weighed and homogenized with PBS to make 20% homogenate. A total of 1 ml of homogenate was added with 0.5 ml of 20% trichloroacetic acid (TCA), then centrifuged at 3000 rpm for 10 minutes. 1 mL of the supernatant was added with 1 ml of 0.67% thiobarbituric acid (TBA), heated at 100°C for 10 minutes, then cooled. The absorption was measured at 532 nm.

Statistical analysis

The data were analyzed with one-way ANOVA followed by Tukey's post-test.

Results and Discussion

Plectranthus scutellarioides leaves extract characteristics

Ethanol extract of *P. scutellarioides* leaves was obtained in the form of concentrated blackish-brown extract and a slightly bitter taste. The characteristics of *P. scutellarioides* leaves extract are presented in Table 1. Phytochemical screening showed that 70% ethanol extract of *P. scutellarioides* leaves contained flavonoids, phenols, saponins, and triterpenoids. The results showed that the total flavonoid level of 70% ethanol extract of *P. scutellarioides* leaves was 12.62 mg QE/g extract. Phytochemical screening showed that the *P. scutellarioides* leaves ethanol extract contained flavonoids, saponins, triterpenoids, and phenols. These compounds have been known to generate antioxidant activity, especially flavonoids.

Table 1: Characteristics of *P. scutellarioides* Leaves Extract

Characteristics	Results
Percentage of yield	18.41 %
Water content	6.71 %
Lost drying	7.84 %

The primary role of flavonoids as an antioxidant is to donate hydrogen atoms to free radicals that cause free radicals to become unreactive or neutral. The hepatoprotective activity of *P. scutellarioides* may be due to this flavonoid compound's presence. To confirm this hypothesis, we examined the total flavonoid content of the extract and the lipid peroxidation in the liver. The result showed that *P. scutellarioides* contains a high flavonoid (12.62 mg QE/g extract). Moreover, the lipid peroxidation test showed a significantly lower number of MDA compared to the negative control (Table 2).

Hepatoprotective Activity of P. scutellarioides leaves extract

Drug-induced liver injury (DILI) has been reported to occur in 19 per 10.000 people a year.[12] DILI can occur through an oxidative stress cascade, where the drug or its metabolites increase the reactive oxygen species (ROS), damaging the mitochondria and triggering immune reactions, thus initiating apoptosis or necrosis and causing cell death.[13] This event eventually causes acute or chronic liver disease.[14] The present study focuses on the approach to prevent liver injury against rifampicin and isoniazid toxicity. Both drugs have been known to cause hepatotoxicity in tuberculosis patients. It causes membrane damage, which increases liver enzymes and bilirubin in the systemic circulation, causing an imbalance in endogenous antioxidants and increased lipid peroxidation.[15]

This study used 50 mg/kg of isoniazid, and rifampicin to induce liver damage in rats. *P. scutellarioides* leave extract was given concurrently with the drugs for 28 days. The hepatoprotective activities were examined by measuring the levels of serum total bilirubin, direct bilirubin, AST, ALT, albumin, liver lipid peroxidation, and histology examination (Figure 1 A – E).

The result showed that the administration of isoniazid and rifampicin for 28 days increased total bilirubin levels by 56.89%, direct bilirubin by 61.37%, and lowered albumin levels by 40.86%. on the other hand, concurrent administration of PSE with the drugs significantly lowered total bilirubin, direct bilirubin, and higher albumin levels than the negative control ($p < 0.05$). The best result seen in PSE 600mg/kg group, where the total bilirubin and direct bilirubin levels, respectively, were 53.45% and 64.98% lower than negative control on day 28, while albumin levels remained stable during the treatment.

Isoniazid and rifampicin caused liver injury, as indicated by the drastic change in all hepatic biochemical parameters since day 14. On the other hand, PSE groups showed improvement in all parameters, except for 150 mg/kg. The results show that PSE treatment groups showed a significant decreased in total bilirubin levels and direct bilirubin, while the albumin level was higher ($p < 0.05$) then negative controls at 300 and 600 mg/kg doses. The same trend was seen in the ALT and AST levels. It was also observed that 600 mg/kg of PSE resulted in stable hepatic parameters and almost no chance compared to day 0 (Figure 2A - C).

The liver enzyme levels in the systemic circulation are indicative of liver cell damage, whereas bilirubin and albumin levels are associated with liver function. Measurement of bilirubin levels in this study was done by the Jendrassik-Grof method, where the bilirubin reacts with Diazotized Sulfanilic Acid (DSA) and forms azobilirubin. Total bilirubin consists of direct (conjugated) and indirect (unconjugated) bilirubin, where glucuronic acid, with the help of uridine diphosphate, conjugates with the bilirubin to form direct bilirubin.[16]

Oxidative stress due to the use of isoniazid and rifampicin also results in leakage of the liver cell membrane and causes the release of transaminase enzymes into the circulation, such as ALT and AST.[17] ALT is present in the cytosol of the liver, while AST is found in the mitochondria of the liver, heart, kidneys, and brain. The damage to these tissues causes an increase in serum ALT and AST levels.[18] PSE group at doses 300 and 600 mg/kg showed a significantly lower level of both AST and ALT than the negative control, indicating its activity in preventing liver cell damage. However, the lowest dose of PSE showed an insignificant effect.

Based on the data, *P. scutellarioides* leaves extract showed hepatoprotective activity in dose depending manner, where the highest dose showed the best activity comparable to Legalon® (equivalent to silymarin 272.15 mg/kg rats BW). Silymarin is a natural compound derived from *Silybum marianum.*[19] It could prevent damage caused by

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tuberculosis drugs through a mechanism of reducing oxidative stress and lipid peroxidation.[20] Silymarin stabilizes the reactive oxygen species (ROS), especially in the liver, and therefore is usually used in hepatitis, hepatic cirrhosis, and other liver disorders.[21]

Histological observations of liver cells show that isoniazid and rifampicin cause inflammation and cell death (necrosis), which is in line with other studies,[11] where these TB drugs induce inflammation characterized by an increase in the number of Kupffer cells. The drugs also cause cell necrosis, as indicated by a shrinking and blackish cell nucleus (pyknotic nucleus).[11] PSE co-administration with isoniazid and rifampicin showed a noticeable histology difference compared to the negative control, especially at 600 mg/kg. PSE could reduce damage in the liver shown by Kupffer cells and the pyknotic nuclei in liver cells (Table 2). Figure 2 depicts a similar observation in Legalon® and PSE 600 mg/kg with normal control, suggesting the hepatoprotective effect of these groups.

In the present study, histological observations were carried out by counting the number of pyknotic nuclei and kupffer cells in liver cells (Table 2). Isoniazid and rifampicin cause the accumulation of toxic substances in liver cells that cause cell death. Microscopically visible changes in the cell nucleus seen as wrinkled or shrunk, denser and dark, with irregular cell boundaries (pyknotic nucleus). Whereas the number of Kupffer cells, which are macrophages in the liver, are increased, showing an inflammatory response.[11] Kupffer cells have an oval shape on the surface of cells in the sinusoid.[22] The identical observation was also seen in humans.

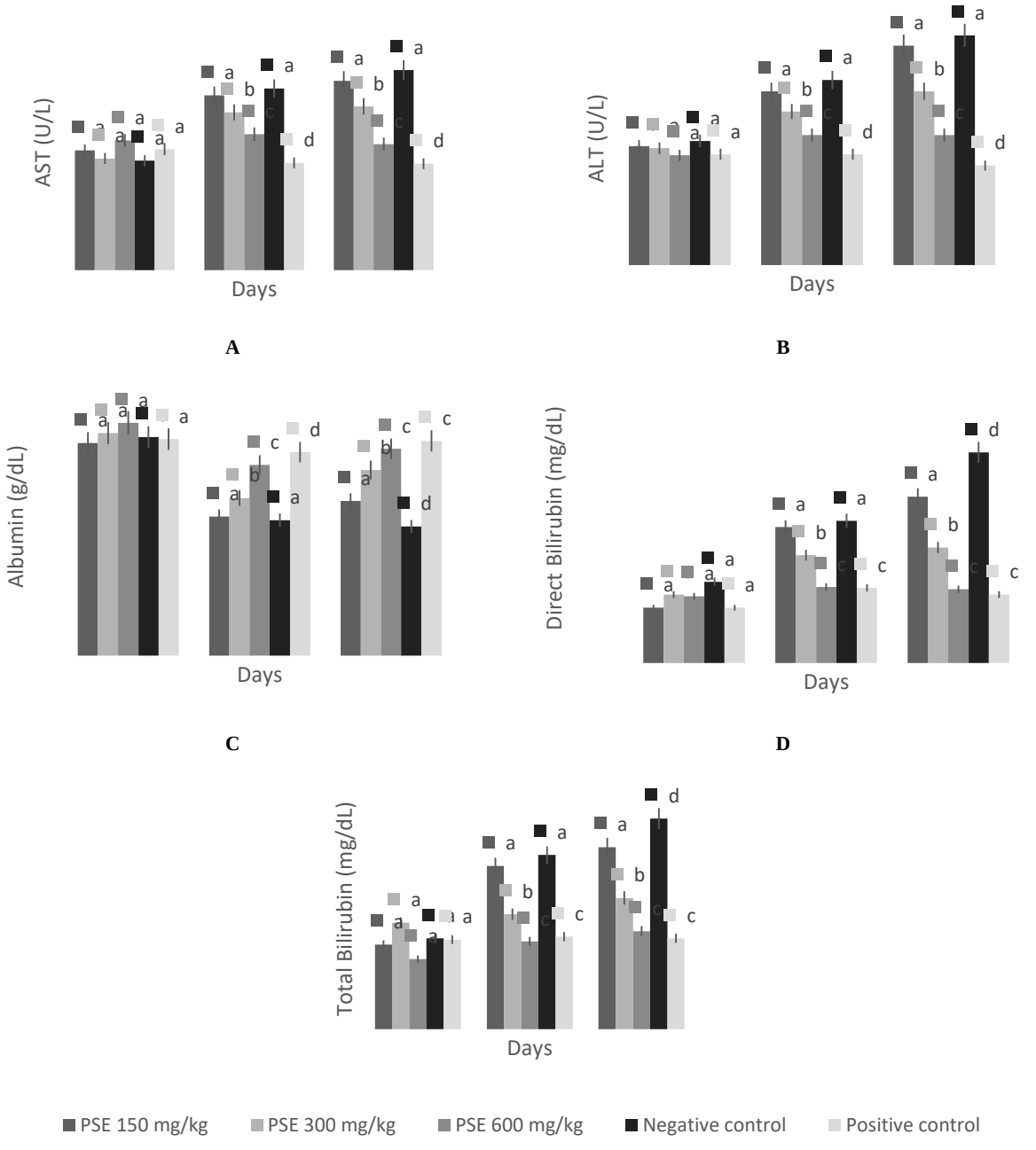

Figure 1: Hepatoprotective effect of *P. scutellarioides* leaves extract day 0 (before treatment), 14 and 28, (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), (C) Albumin Levels (D)Direct bilirubin, (E)Total bilirubin. The means in each day that do not share a letter are significantly different ($p < 0.05$)

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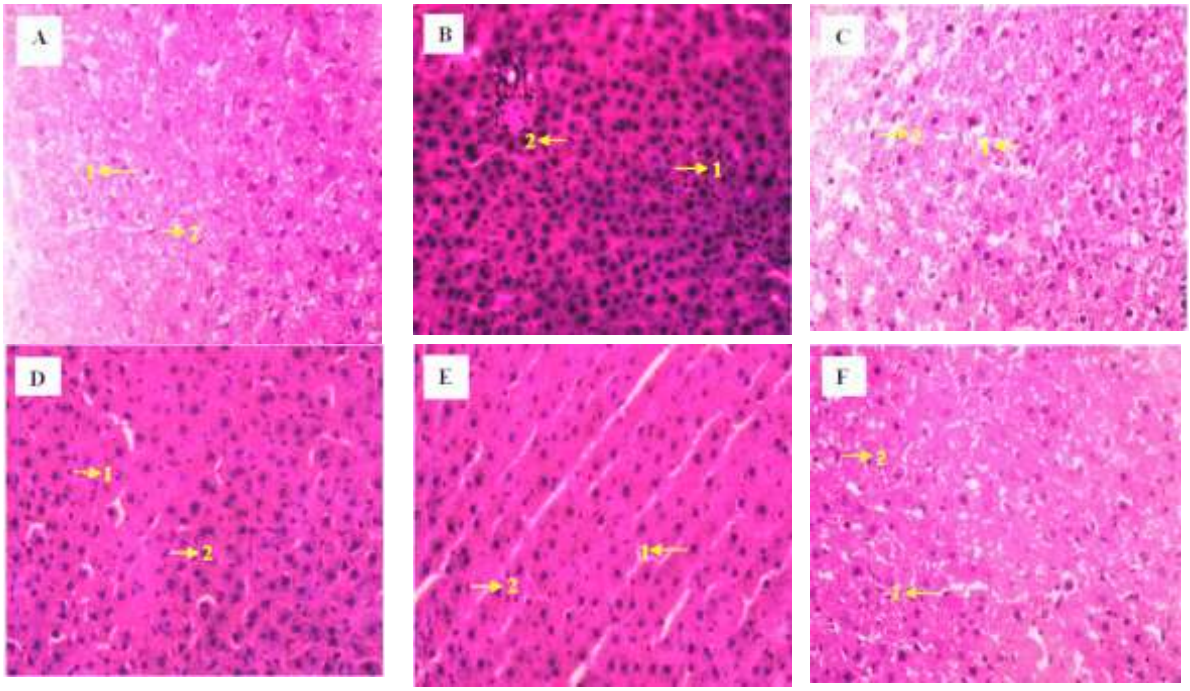

Figure 2: Histological Observation of Livers (A) Normal control (B) Negative control (C) Legalon® (D) PSE 150 mg/kg (E) PSE 300 mg/kg (F) PSE 600 mg/kg (1) Pyknotic nucleus (2) Kupffer Cells (400x), PSE (*Plectranthus scutellarioides* Leaves extract).

Table 2: The Histological and Chemical Examination of The Rats Liver

Groups	Pyknotic nuclei X ± SD, n=4	Kupffer cells X ± SD, n=4	MDA X ± SD, n=4
PSE 150mg/kg	113.0 ± 11.2 [d]	75.32 ± 6.21 [d]	19.51 ± 0.42
PSE 300 mg/kg	93.00 ± 4.69 [e]	58.45 ± 7.84 [e]	17.70 ± 0.45 [b]
PSE 600 mg/kg	71.55 ± 11.1 [c]	41.25 ± 3.10 [c]	15.63 ± 0.69 [d]
Legalon®	62.80 ± 8.03 [c]	31.70 ± 2.70	13.04 ± 0.83
Negative control	136.2 ± 7.99 [b]	88.00 ± 7.25	21.90 ± 0.84 [e]
Normal control	47.90 ± 7.95[a]	21.80 ± 3.29 [a]	12.74 ± 1.07 [c]

The same subscript in each column shows comparable results (p>0.05), PSE (*Plectranthus scutellarioides* Leaves extract)

Liver tissue biopsy in patients taking these drugs shows an increase in inflammatory cells, especially histiocytes and neutrophils.[23] The number of pyknotic nuclei and kupffer cells in the group that received PSE showed mild liver damage compared to the negative control group, especially at a dose of 600 mg/kg.

Studies have shown several potential plants as hepatoprotector. Another species of *Plectranthus*, *P. amboinicus* also showed hepatoprotective effect at 600 mg/kg doses in paracetamol-induced rats.[24] *Plectranthus* genus exhibits hepatoprotective activity at the same dose, despite the different drugs used to induce liver injury. Despite PSE's success in preventing liver damage, satisfactory results were only seen at high doses. Compared to other studies, such as *Telfairia occidentalis* at a dose of 500mg/kg[25] and *Alpinia Officinarum at* a dose of 400 mg/kg,[26] showed a lower dose as hepatoprotector against rifampicin and isoniazid. The biological activity of *P. scutellarioides* leaves extract was due to the complexity of the chemical compounds contained therein. The fractions/isolates of this plant should be further studied so that the development of *P. scutellarioides* leaves as hepatoprotector can be optimal.

Conclusion

Based on liver enzyme levels and liver function tests, PSE showed beneficial effects in preventing liver damage due to isoniazid and rifampicin hepatotoxicity. It can be concluded that PSE has hepatoprotective potential, especially at a dose of 600 mg/kg.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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