INTRODUCTION

Piperine is known to have many pharmacological benefits such as anti-hypertensive, antioxidants, anti-inflammatory, anti-asthmatic, and hepatoprotective. Piperine has been associated with numerous of toxic effects. Piperine in Piper longum has a toxic effect on DLA cells (Dalton Lymphoma Ascites) and EAC cells (Ehrlich Ascites Carcinoma). Many studies have reported the pharmacological benefits of piperine, but scientific studies of the subchronic toxicity of piperine have not been reported.

Toxicity testing on animals can be useful for detecting biochemical, physiological, and pathological reactions. A compound is said to be toxic if it causes changes in physiology, neurology, hematology, and clinical biochemistry in test animals, as well as changes in body organs not only macro-pathologically but also histologically. Data from the toxicity test can be used to determine the degree of danger posed by the test preparation when exposed to humans, allowing the dosage for human use to be determined.

Before it can be used as medicine, both herbal and non-herbal medicinal ingredients must be tested for safety. To ensure the safety of a substance, a toxicity test is performed. A toxicity test determines whether a substance has a toxic effect. Improper use of natural ingredients has the potential to cause a variety of undesirable side effects. Not only drugs but also natural ingredients pose a significant risk of causing organ damage. The toxicity test was divided into three categories: acute, sub-chronic, and chronic. Toxic effects can occur in animals' vital organs used as toxicity test materials, such as the kidneys, liver, and lungs. The kidneys have many functions, including excreting many foreign compounds that enter the body, such as drugs, food additives, pesticides, and other non-nutritive exogenous materials. The lung is one of the vital organs where the exchange of oxygen and carbon dioxide takes place, which is critical in the body’s metabolic processes. The lung also receives a lot of blood flow and plays an important role in maintaining the body’s acid-alkaline balance. The occurrence of disorders or damage to the pulmonary organs can have an impact on the body’s metabolic processes. The liver is the body's largest organ and arguably the most important organ for protein production and detoxification.

ABSTRACT

INTRODUCTION: Piperine is a compound that has many health benefits, but piperin has toxic effects that endanger the human body. The purpose of this study was to determine the toxic dose of subchronic piperine administration using histology of the liver, kidneys, and lungs of mice (Mus musculus L.).

Method: This is a quasi-experimental research with posttest only control group design. Thirty male BALB/c mice were divided into five groups. Piperine was administered orally to the mice. The normal control group and four treatment groups at dosage of 17.5, 35, 70, and 140 mg/kg body weight for 21 days. At the day-22, the liver, kidneys, and lungs were removed and prepared using the HE staining technique. Scores of liver, kidney, and lung damage are recorded. The data was analyzed using one-way ANOVA with the post hoc Tukey test.

Result: The liver histological damage score after administration of piperine doses of 35, 70, and 140 mg/kg significantly different from the control group. The kidney and lung damage scores after 140 mg/kg administration showed significant differences from the control group. The conclusion is piperine at dosage of 35, 70, and 140 mg/kg was toxic to the liver, and piperine at doses of 140 mg/kg was toxic to the kidney and lung histology of BALB/c mice.

Conclusion: Piperine should not be used at doses of 35 mg/kg body weight or larger.

Keywords: Piper nigrum, subchronic toxicity, mice, histology.

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sub-acute toxicity study at a dose rate of 100 mg/kg for 7 days.13
The purpose of this study was to determine the toxic dose of subchronic piperine 17.5; 35; 70; and 140 mg/kg body weight dose of piperine every day for 21 days administration using histology of the liver, kidneys, and lungs of mice (Mus musculus L.).

METHODS

Research design
This is a quasi-experimental study with a posttest-only control group design. The test material was the ethanol extract of Piper nigrum L (white pepper) in the form of a piperine compound. Piperine is made in several doses with corn oil solvent.

Animals and treatment
Thirty male BALB/c mice, aged three months and weighing 35-45 g, were divided into five groups. For each group, mice were housed in 40x30x15 cm plastic cages. For one week of acclimation, mice were fed regular mouse pellets and water ad libitum. Mice were divided into five groups of six mice each group, which were randomly chosen. The normal control group and four treatment groups received 17.5, 35, 70, and 140 mg/kg body weight doses of piperine orally every day for 21 days.14 This dosage is based on a previous study conducted by Piyachaturawat et al.11 The mice in the control group were only given corn oil orally once a day. The mice were sacrificed on the 22nd day. The kidneys, lungs, and liver were fixated in 10% neutral-buffered formalin before being thin sectioned. Hematoxylin and Eosin (H&E) were used to stain some sections.

A light microscope with a 400x magnification was used to examine the mice corpuscle section in 5 fields of view in the renal corpuscle diameter.15 A light microscope with a magnification of 400x was used to examine lung preparations in five fields of view. The thickening of the interalveolar septum was observed in the histology.16

The mice livers were examined by assessing the damage scores in the degeneration and necrosis of the hepatocytes using the modified reference scoring method and observing the presence of damage to 100 liver cells (hepatocytes) in one field of view, as shown in Table 1.17 A 400x magnification light microscope was used to examine hepatocytes in 5 fields of view.

Statistical analysis
Liver, kidney, and lung damage scores were expressed as mean ± standard deviation. One-way ANOVA with the post hoc Tukey test was used to analyze the data.

RESULTS

As shown in Table 2, the benchmarks in this piperine subchronic toxicity test are changes in the degree of liver damage, the diameter of the renal corpuscle, and the thickness of the septum interalveolar.

Kidney toxicity
The results of kidney histology observations in mice (Mus musculus L.) revealed that the average renal corpuscle diameter of the control group was 67.13±4.14 µm, which increased in the piperine group with a dose of 17.5 mg/kg BW to 68.14±6.27 µm. As shown in Figure 1, the piperine group with a dose of 35 mg/kg BW had the smallest mean renal corpuscle diameter compared to other groups, namely 62.54±4.47 µm, which increased at 70 mg/kg piperine, namely 63.57 ± 8.9 m, and increased again at the highest piperine dose of 140 mg/kg, namely 76.08±5.14 m.

The data analysis revealed that piperine administration had a significant effect on changes in the renal corpuscle diameter of mice (p=0.006). The Post Hoc LSD Test revealed that the piperine group with a dose of 140 mg/kg BW was significantly different from the control group and the other piperine dose group (p<0.05). When compared to the control group, the piperine group with a dose of 35 mg/kg BW, and the piperine group with a dose of 70 mg/kg BW, the piperine group with a dose of 17.5 mg/kg BW was not significantly different (p>0.05). When compared to the control group and the piperine group with a dose of 70 mg/kg BW, the piperine group with a dose of 35 mg/kg BW was not significantly different (p>0.05). When compared to the

Table 1. Liver cell damage scoring.17
| Score | Criteria |
|-------|----------|
| Score 0 | One power filed did not find degeneration and necrosis |
| Score 1 | One power filed was found 1-20% degeneration and necrosis |
| Score 2 | One power filed was found 21-50% degeneration and necrosis |
| Score 3 | One power filed was found 51-75% degeneration and necrosis |
| Score 4 | One power filed was found more than 75% degeneration and necrosis (heavy damage) |

Table 2. Scores of kidneys, livers, and lungs damage of mice after given of piperine orally.

| Group | Kidney (diameter renal corpuscle (µm)) | Liver (degree of liver damage) | Lungs (thickness of septum inter-alveolar (µm)) |
|-------|----------------------------------------|-------------------------------|-----------------------------------------------|
| Control | p: 0.006 | p: 0.000 | p: 0.001 |
| Piperine dosage 17.5 mg/kg BW | 67.13±4.14<sup>a</sup> | 0.96±0.15<sup>a</sup> | 4.41±0.41<sup>a</sup> |
| Piperine dosage 35 mg/kg BW | 68.14±6.27<sup>a</sup> | 1.46±0.43<sup>b</sup> | 4.84±0.75<sup>a</sup> |
| Piperine dosage 70 mg/kg BW | 62.54±4.47<sup>a</sup> | 1.90±0.20<sup>b</sup> | 5.81±1.96<sup>a</sup> |
| Piperine dosage 140 mg/kg BW | 63.57±8.90<sup>a</sup> | 2.00±0.28<sup>b</sup> | 6.29±2.64<sup>a</sup> |
| Piperine dosage 140 mg/kg BW | 76.08±5.14<sup>a</sup> | 2.30±0.41<sup>a</sup> | 9.10±2.04<sup>a</sup> |

Note: numbers followed by the same letter showed no significant difference (p≥0.05)
control group, the piperine group at a dose of 17.5 mg/kg BW, and the piperine group at a dose of 35 mg/kg BW, the piperine group at a dose of 70 mg/kg BW was not significantly different (p>0.05).

The piperine treatment group with a dose of 140 mg/kg BW had a significant difference in histological observations of renal corpuscle diameter, which was 76.08±5.14 m. This indicates that the piperine dose of 140 mg/kg BW is toxic to the kidneys.

Liver toxicity
The mean score of liver cell damage in the control group was 0.96±0.15, then increased in the piperine group with a dose of 17.5 mg/kg BW, namely 1.46±0.43, then increased in the piperine group with a dose of 35 mg/kg BW, namely 1.90±0.20, then increased in the piperine group with a dose of 70 mg/kg BW. As shown in Figure 2, the group that received 140 mg/kg BW of piperine had the highest mean score of liver damage in mice, namely 2.30±0.41.

The data analysis revealed that piperine administration had a significant effect on the histological changes in the liver cells of mice (p=0.000). Tukey's Post Hoc Test was used to continue data analysis, which revealed that the control group had a significant difference in the piperine group with doses of 35 mg/kg BW, 70 mg/kg BW, and 140 mg/kg BW, and there was a significant difference between the piperine group with a dose of 17.5 mg/kg BW and the piperine group giving a dose of 140 mg/kg BW.

Lung toxicity
The findings of this study show that subchronic administration of piperine at various doses can cause changes in the thickness of the interalveolar septum of the mice's lungs (Mus musculus L). The thickness of the interalveolar septum increased with increasing piperine dose. The piperine group with the lowest dose of 17.5 mg/kg BW had the thinnest interalveolar septum thickness, while the piperine group with the highest dose of 140 mg/kg BW had the thickest interalveolar septum thickness. Table 2 shows the mean thickness of the interalveolar septum.

The lungs histology of mice (Mus musculus L) revealed that the control group had the thinnest interalveolar septum with an average thickness of 4.41±0.41 m, followed by the piperine treatment group with a dose of 17.5 mg/kg BW (4.84±0.75 m) in the piperine treatment group. The piperine treatment group had the thickest interalveolar septal thickness (9.10±2.04 m) with a dose of 140 mg/kg BW (7.5 mg/kg BW (5.81±1.96 m), the piperine treatment group with a dose of 70 mg/kg BW (6.29±2.64 m), and the piperine treatment group with a dose of 140 mg/kg BW. Piperine administration, in general, can cause an increase in the thickness of the interalveolar septum in mice, as shown in Figure 3.
Statistical analysis revealed that piperine administration had a significant effect on the thickness of the interalveolar pulmonary septum in mice \((p=0.001)\). Using the Post Hoc LSD Test, it was discovered that the piperine treatment group at a dose of 140 mg/kg BW differed significantly from the control and treatment groups \((p<0.05)\). The piperine treatment group at 17.5 mg/kg BW did not differ significantly \((p>0.05)\) from the control group, the piperine treatment group at 35 mg/kg BW, and the piperine treatment group at 70 mg/kg BW. When compared to the control group and the piperine treatment group with a dose of 70 mg/kg BW, the piperine treatment group with a dose of 35 mg/kg BW did not provide a significant difference \((p>0.05)\). When compared to the control group, the 70 mg/kg BW treatment group did not significantly differ \((p>0.05)\).

The thickness of the interalveolar septum increased in all treatment groups, according to the findings. The piperine group with a dose of 140 mg/kg BW had a significant difference in histological observations of the thickness of the interalveolar septum, which is 9.10±2.04 m. These findings suggest that piperine causes lung alveolar damage.

DISCUSSION

Kidney toxicity

Piperine can be found in serum at a concentration of 0.016 percent 1-10 hours after administration. This study revealed that piperine enters the bloodstream and, in high doses, can cause organ damage. The findings are consistent with the discovery that piperine can enter the circulation and affect histology in the kidneys. Piperine is absorbed in a rate of 97%, with the remaining 3% excreted as piperine in feces. Piyachaturawat et al. previously reported that piperine could cause severe hemorrhage and edema along the gastrointestinal tract, ulceration of the stomach with necrotic epithelial cells, and desquamation in mice. Piperine has an effect on organs that are in direct contact, according to these studies.13

Another change in the kidney histology of mice \((Mus musculus L)\) is a change in the thickness of the Bowman's space. Bowman space thickness increases and decreases with piperine dose, but there is no significant difference between changes in Bowman space thickness. A swollen glomerulus changes the shape of Bowman capsule proliferation, resulting in adhesion between the glomerulus and Bowman's connective tissue caused by exogenous or endogenous stimuli that result in vascularization. Inflammation can be caused by microorganisms, mechanical trauma, chemical substances, or physical influences. The body's natural response to tissue damage is inflammation. Many chemical mediators, such as histamine, 5-hydroxytryptamine (5HT) or serotonin, chemotactic factors, bradykinin, leukotrienes, and prostaglandins, are released locally during inflammation. Acute or chronic inflammation can occur. After the mediators are activated, the inflammation will resolve. Chronic inflammation occurs when the source of the inflammation cannot be eliminated or when antigens are exposed repeatedly, causing tissue damage and loss of function.18

The glomerulus is a kidney component that frequently experiences abnormalities due to immune complex deposition, thrombosis, embolism, and viral infections in the glomerular component. Glomerular damage is characterized by necrosis as well as morphological and functional changes in the cell membrane. Leukocyte infiltration as well as morphological proliferation. Glomerular swelling occurs as a result of endothelial proliferation and leukocyte infiltration as a result of immune complex deposition caused by the inflammatory response.18

Meanwhile, the study discovered that piperine doses of 17 mg/kg BW, 35 mg/kg BW, and 70 mg/kg BW did not show any significant differences in the control group. Piperine doses of 17 mg/kg BW, 35 mg/kg BW, and 70 mg/kg BW are thought to be safe for consumption and have not affected the increase in the overall diameter of the renal corpuscle. The detoxification process in the liver before elimination in the kidneys may reduce the toxicity risk of piperine.20

Figure 3. Histological feature of the interalveolar septal pulmonary mice using 400x magnification in the control group (C) shows in the histological features of the thin interalveolar septum; while the piperine treatment group dose 17.5; 35; 70; 140 mg/kg BW (A-D) shows an increase in the thickness of the interalveolar septum along with the increase in piperine dose compared to the control group. Slope: red arrows indicate the interalveolar septum.
capsule and narrowing of the Bowman space.\textsuperscript{21} The glomerulus swells, causing the Bowman space to become thinner.\textsuperscript{22}

Toxic nephropathy can occur when the kidneys are exposed to toxic substances, affecting the glomerulus, tubules, vascular tissue, and interstitial tissue. One of the causes of chronic renal failure is toxic nephropathy. Chronic renal failure is a clinical condition characterized by progressive and reversible kidney damage.\textsuperscript{22}

Piperine given orally at doses of 50, 100, and 200 mg/kg BW induced by lead acetate for 65 days can reduce nephrotoxicity and become nephroprotective in rats’ kidneys, according to research published in 2017.\textsuperscript{23} This contradicts the findings of a previous study in which piperine was found to be nephrotoxic to the kidneys of mice. This can be caused by differences in the dose given to the test animals as well as the length of time the test animals were exposed.

**Liver toxicity**

Significant differences in the liver histology of mice (\textit{Mus musculus L}) were observed between the control group and the piperine groups at doses of 35 mg/kg BW, 70 mg/kg BW, and 140 mg/kg BW. Piperine compounds were found in the body’s circulation after 1-10 hours of piperine administration, according to Bhat et al. This demonstrates that piperine when ingested, can affect organs and even cause damage if high doses are administrated. This statement is consistent with the findings of this study, which found that when piperine enters the circulation, it can harm the test animals’ organs. Piperine, according to Piyachaturawat et al., can cause severe hemorrhage and edema along the gastrointestinal tract, ulceration in the stomach region covered with squamous cells, moderate enteritis in the small intestine, and histological changes in the bladder and adrenal glands. As a result, piperine can cause both local and systemic damage in test animals.\textsuperscript{13}

According to this study, the pulmonary organ that is not in direct contact with the piperine compound has histological damage in the form of an increase in the thickness of the interalveolar septum.

Damage to the lungs’ alveoli can cause an inflammatory response in the lung tissue. Acute inflammation causes redness, leukocyte accumulation, the release of inflammatory mediators, and increased blood flow, resulting in extravasation of fluid and edema in tissue that has been injured or infected with pathogens. Acute inflammation that is not controlled can progress to chronic inflammation.\textsuperscript{29,30} Chronic inflammation causes the production of substances that promote the formation of new tissue, such as collagen and blood vessels.\textsuperscript{28} The presence of chronic inflammation of the pulmonary alveoli is indicated by an increase in fibrous tissue in the alveolar septum and interstitial edema, which causes an increase in alveolar septum thickness. Because the alveolus contains many capillaries, edema is easily caused by increased capillary epithelial permeability.\textsuperscript{28}

Inflammatory processes and oxidative stress in the lungs can cause histological changes in lung tissue. Oxidative stress is caused by an imbalance between oxidants
and antioxidants, in which the body is unable to neutralize free radicals that accumulate in the body.\textsuperscript{31} Exogenous and endogenous sources of free radicals exist in the body. It can be obtained exogenously from cigarette smoke, pollutants, drugs, and food. Reactive oxygen species are normally produced by cellular metabolic processes in endogenous sources (ROS).\textsuperscript{32} Toxins are produced by oxidative stress caused by reactive oxygen species (ROS) via redox reactions and fatty acid oxidation.\textsuperscript{33} According to Grinevicius et al., giving \textit{Piper nigrum} L ethanol extract could increase the body’s production of ROS.\textsuperscript{34} Oxidative stress may trigger local immune responses, increase the number of macrophages and neutrophils in lung tissue, and impair lung function.\textsuperscript{35} Damage to the alveolar epithelium can result in the release of cytokines and growth factors that stimulate extracellular matrix secretion and the proliferation of myofibroblasts, particularly Transforming Growth Factor (TGF-), Platelet-Derived Growth Factor Receptor (PDGFR-), and Tumor Necrosis Factor (TNF-), which are thought to stimulate the formation, proliferation, and accumulation of fibroblasts, resulting in extracellular matrix deposition.\textsuperscript{36}

Piperine has been shown in previous studies to have cytotoxic activity. Piperine, at a dose of 250 g/ml, has cytotoxic effects on DLA (Dalton Lymphoma Ascites) and EAC (Ehrlich Ascites Carcinoma) cells via an immunomodulatory mechanism. Immunomodulators can activate cytotoxic effector cells such as cytotoxic T lymphocytes, natural killer (NK), macrophages, and lymphocytes.\textsuperscript{36,37}

According to Bang et al., piperine administered orally at doses of 20 and 100 mg/kg for 8 days had an anti-inflammatory effect by inhibiting IL-6 in a mouse model of carrageenan-induced arthritis.\textsuperscript{38} In contrast to the findings of this study, a later study found piperine to be a proinflammatory agent. This can be caused by differences in the dose of administration, the route of administration, and the length of exposure. The study also stated that piperine could inhibit IL-6, while there were many other inflammatory-causing factors such as TNF-, IL-1, IL-6, IL-10, TGF, NO, and ROS.\textsuperscript{39}

The limitation of this study was the data of this study only observed the histological aspects of the kidneys, lungs, and liver. This study did not measure parameters in blood or other organs.

**CONCLUSION**

This study demonstrated that administration of 35; 70 and 140 mg/kg doses of piperine had a toxic effect on the liver, and 140 mg/kg dose of piperine had a toxic effect on the kidney and lung histology of BALB/c mice.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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**ETHICS APPROVAL**

All experimental protocols have been approved by the Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, No. 098/EP-FKIK-UMY/III/2019, 100/ EP-FKIK-UMY/III/2019, and 101/EP-FKIK-UMY/III/2019.

**AUTHOR CONTRIBUTORS**

SNNM contributed to the design of the research variables, analysis of data, and writing the manuscript. ST & PNA contributed to the basic concept and design of the study. KNI, RN, and AP contributed to performing the experiment, data collection, writing report articles. All authors have read and approved the final manuscript.

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