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Liver Histopathological Analysis in The Acute Toxicity Test of Shallot (*Allium cepa* L.) Peel Extract in Rats (*Rattus norvegicus*)

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Abstract

Shallot (*Allium cepa* L.) contains bioactive compounds with antioxidant effects. In addition to the tuber, the shallot's peel is a rich source of flavonoids with demonstrated capacity to mitigate oxidative stress. Prior studies have evaluated the antioxidant efficacy of shallot peel in ameliorating organ damage and have established its maximum effective dose. The subsequent step required for its potential therapeutic application is toxicity test. This study aimed to analyze the liver histopathological changes in the acute toxicity test of shallot peel extract (SPE) in rats based on OECD TG 420. This research was an experimental with a post-test-only control group design. Five female rats were used as the control group administered dimethyl sulfoxide (DMSO), while 5 female rats were used in the treatment group administered SPE at 5,000 mg/kg body weight (BW) (one of them had been used for a preliminary test with the same dosage). On day 15, a necropsy was conducted, followed by histopathological observation of the hematoxylin-eosin (HE)-stained liver histopathological slide. The damage to hepatocytes was evaluated using Manja Roenigk criteria. The average histopathological score per liver cell of the control group was 1.226 ± 0.0065 and the treatment group was 1.235±0.0079. The Mann-Whitney test showed that the liver histopathological score of the treatment group was not different from that of the control group ($p>0.05$). It can be concluded that SPE 5,000 mg/kg does not show acute toxic effects in rats, with LD_{50} estimated at >5,000 mg/kg according to OECD standards.

Keywords: acute toxicity, histopathological, liver, shallot peel

Introduction

Shallot (*Allium cepa* L.) ranks as the second most significant horticultural crop in Indonesia, known for its potent antioxidant properties attributed to its high flavonoid content (Kementerian Pertanian, 2023). The total flavonoid concentration in shallot skin extract is measured at 228.1 mg QE/L (Rahima et al., 2022). Often regarded as waste, shallot peels are found to contain a substantial number of flavonoid compounds, particularly quercetin, which is present at levels 3-5 times higher than in the tubers. The flavonoid content in shallot peel extract (SPE) exhibits a scavenging ability that disrupts free radical chain reactions (Fuentes et al., 2020; Mobin et al., 2022). It also enhances the antioxidant capacity of cells by modulating the expression of the Nrf2 gene, leading to increased levels of cellular antioxidant enzymes (Xu et al., 2019).

Previous studies on SPE have investigated its effectiveness as an antioxidant in repairing organ damage and have established its maximum effective dose. One study demonstrated that SPE at a dose of 1,200 mg/kg body weight (BW) neutralized oxidative stress caused by diazinon, as evidenced by a reduction in liver malondialdehyde (MDA) levels and a decrease in the number of activated Kupffer cells to levels comparable to the normal group (Rahima et al., 2022). Additionally, another study indicated that SPE improved liver damage in diazinon-induced rats, as shown by a decrease in liver cell damage scores (Cahya et al., 2022). Research on the respiratory system revealed that the maximum effective doses of shallot peel infusion to prevent bronchial epithelium thickening and cilia shortening were 1,275.4 mg/kg BW and 1,325.8 mg/kg BW, respectively (Bintang et al., 2024). Furthermore, the amelioration of kidney damage was found to be moderately correlated with a maximum effective dose of

shallot peel infusion of 1,359 mg/kg BW for mitigating kidney damage in diazinon-induced rats (Bi'izzyk et al., 2024).

Before SPE can be considered safe for consumption, particularly as a functional food or therapeutic agent, it is essential to evaluate its potential toxicity when administered in higher doses. Toxicity test is a method performed on experimental animals to identify the toxic effects of a test substance on the body's systems (Madihah et al., 2017). The first toxicity test needed to be carried out is acute toxicity test to detect toxic effects that occur within a short period (Badan Pengawas Obat dan Makanan, 2022; Rachmawati & Ulfa, 2018). At high concentrations and under specific conditions, dietary phenolic compounds can exhibit pro-oxidant and cytotoxic effects. Their pro-oxidant activity is influenced by factors such as phenolic type, pH, metal ions (e.g., Cu^{2+}, Fe^{2+}), oxygen presence, and solubility. This activity leads to ROS generation, lipid peroxidation, DNA damage, and apoptosis. However, these pro-oxidant effects may also contribute beneficially to cancer prevention and the mitigation of oxidative stress-related disorders (Guneidy et al., 2022). Given their potential pro-oxidant effects at high doses, it is essential to evaluate the safety of these compounds through standardized toxicity test, such as the Organisation for Economic Co-operation and Development Test Guideline 420 (OECD TG 420) acute oral toxicity guideline (Madihah et al., 2017; OECD, 2001; Sulastra & Khaerati, 2020).

The presence or absence of acute toxicity of a substance in experimental animals is assessed by evaluating the heart, lungs, liver, kidneys, and spleen (Ayun et al., 2021). The liver is the body's primary metabolic organ and plays a crucial role as a detoxification organ, processing and eliminating harmful substances from the body, such as toxic compounds and metabolic waste, through a process known as detoxification. Consequently, the liver is susceptible to damage and functional decline due to exposure to foreign substances (Merdana et al., 2019; Wahyuni et al., 2017). Microscopic changes in the liver due to the toxic effects of drugs often can be observed as inflammatory cell infiltration, hepatocytes degeneration and necrosis (Taek et al., 2019). Currently, there is limited or no data regarding the acute toxicity profile of SPE, particularly its impact on liver histopathology, hence this study aims to analyze the liver histopathological changes in the acute toxicity test of SPE in rats (*Rattus norvegicus*).

Methods

The Study Design

This research is an experimental design with a post-test-only control group. The study was conducted from November 2023 to April 2024 and received approval from the Ethics Commission of the Faculty of Medicine at University of Jember, under letter number 2132/UN25.1.10.2/KE/2024. The preparation of shallot peel extract was conducted at the Biology Laboratory, Faculty of Pharmacy, University of Jember. Acclimatization, treatment, and euthanasia of the experimental animals were performed at the Experimental Animal House, Faculty of Dentistry, University of Jember. Meanwhile, histopathological slides were made and observed at the Histology Laboratory, Faculty of Medicine, University of Jember.

The Preparation of SPE

The shallot peels used in this research were obtained from Tanjung Market in the Kaliwates District of Jember Regency (Biru Lancor, *Allium cepa* L., var. ascalonicum Back). Shallot peels were soaked in a 2% salt solution based on the total volume of the soaking water, and subsequently sun-dried. Next, the dried peels were ground using a blender and sifted to obtain smaller particles. The extraction was performed using the maceration method with 96% ethanol as the solvent. Shallot peel extract was obtained by soaking 200 g of shallot peel powder in 96% ethanol until reaching a total volume of 2 L for 24 hours. The ratio of the shallot peel powder to the solvent was 1:10. The extraction process was repeated three times, using fresh solvent for each repetition. The resulting extract was then filtered using Whatman filter paper number 2 to separate the filtrate from the residue. Finally, the filtrate was evaporated at a temperature of 50°C to obtain a thicker extract. This procedure is in accordance with previous studies with modifications (Cahya et al., 2022).

The resulting extract was semi-solid in form and therefore required dissolution in dimethyl sulfoxide (DMSO) prior to administration to experimental animals. A stock solution of SPE at a dose of 2,000 mg/kg BW was prepared by dissolving 2,000 mg of the extract in 5 mL of DMSO at a specific concentration (equivalent to 400 mg of SPE dissolved in 1 mL of DMSO at a given concentration). A 1% DMSO solution was prepared by mixing 0.1 mL of 100% DMSO with distilled water to a final volume of 10 mL. Similarly, 2% and 3% DMSO solutions were prepared by mixing 0.2 mL and 0.3 mL of 100% DMSO with distilled water, respectively, each adjusted to a final volume of 10 mL. A total of 400 mg of SPE was dissolved in 1% DMSO to a final volume of 1 mL. If the extract did not completely dissolve, a higher DMSO concentration (2% or 3%) was used (Joshi & Adhikari, 2019) In this study, SPE was found to be soluble in 2% DMSO.

The Acute Toxicity Test of SPE

This study used 10 female Wistar rats *(Rattus norvegicus)* aged 8-12 weeks, weighing 80-120 g, not pregnant, and nulliparous. Shallot peel extract was administered in a single dose orally based on OECD TG 420 fixed-dose procedures. This test involved one rat in the sighting study with a single dose of 2,000 mg/kg BW. If the rat experienced mortality, the sighting test dose would be reduced to 300 mg/kg BW and adjusted accordingly following OECD TG 420 guidelines. If the rat did not show any signs of death, the dose could be increased to 5,000 mg/kg BW involving another rat. If there were no fatalities at this dose during the sighting test, the main test could commence at 5,000 mg/kg BW, involving the initial rat plus four additional rats. Moreover, in this main test, 5 rats were used as controls administered a single dose of DMSO, adjusted according to the gastric capacity of the rat, specifically 1 mL/100 g of BW(OECD, 2001) The administration of SPE and DMSO was done orally through a probe (sonde). If during the main test with a dose of 5,000 mg/kg BW, there are ≥2 fatalities among the test animals, the dose must be reduced to 2,000 mg/kg BW. Conversely, if there is ≤1 fatality at the 5,000 mg/kg BW dose, the substance can be classified as non-toxic (OECD, 2001).

The Histopathology Preparation and Analysis

On day 15, the rats of the preliminary and main tests were anesthetized with xylazine and ketamine via intramuscular injection, followed by organ necropsy. The right liver lobe was sliced using a microtome to a thickness of 3 microns and then stained using the hematoxylin-eosin (HE) staining method. The histopathological slides of the rat liver were observed using a Leica DM500 binocular microscope with 400X magnification. The images were captured using AmScope FMA050 (Fixed Microscope Adapter). Microscopic observations were conducted on 5 fields of view, with a total of 20 cells examined for each field of view, performed in zone 3 of the right lobe of the liver using a systematic zig-zag pattern based on the Manja Roenigk scoring criteria (score 1: normal, score 2: parenchymal degeneration, score 3: hydropic degeneration, and score 4: necrosis) by two observers (Cahya et al., 2022).

The Statistical Analysis

To evaluate the consistency among histopathology observers, a Cronbach's alpha test was conducted. This test aims to determine whether the same interpretations and concepts were applied to the items within a research assessment or questions, thereby estimating reliability to ensure the validity of the test. The Cronbach's alpha value ranges from 0 to 1, with $\alpha > 0.7$ generally regarded as acceptable in most studies (Bonasia et al., 2015; Singla et al., 2017). The results of the histopathological liver scoring for the control and treatment groups were analyzed using the Mann-Whitney test.

Results

In the main test using SPE at a dose of 5,000 mg/kg BW, no signs of toxicity either behavioral or physiological signs or death were observed in the rats during the 14-day observation period. Therefore, in the main experiment, SPE was administered at a dose of 5,000 mg/kg BW to 1 initial rat and 4 additional rats. Five rats in the control group were given DMSO in a single dose. The evaluation for all rats over 14 days did not show any signs of toxicity or death. The microscopically qualitative observations of the liver in both the control and treatment groups indicated that most liver cells displayed normal features, such as a polygonal shape, uniform red cytoplasm, distinct cell membrane boundaries, and no signs of enlargement. A minimal number of hepatocytes in both groups exhibited signs of parenchymal degeneration, which included swollen cells, cloudy cytoplasm with granules, and hydropic degeneration characterized by pale (clear) cytoplasm, vacuolization, and cell swelling. No necrosis was observed in either group (Figure 1).

The average histopathological score per liver cell from 100 cells of each rat in the control and treatment groups, obtained from two observers, are presented in Table 1. The Cronbach's alpha test for hepatocyte scores resulted in a value of 0.939 ($\alpha > 0.7$), indicating that the data were reliable. The Mann-Whitney test showed a significance value of 0.071 ($p > 0.05$), indicating no significant difference of the liver histopathological score between the control and treatment groups.

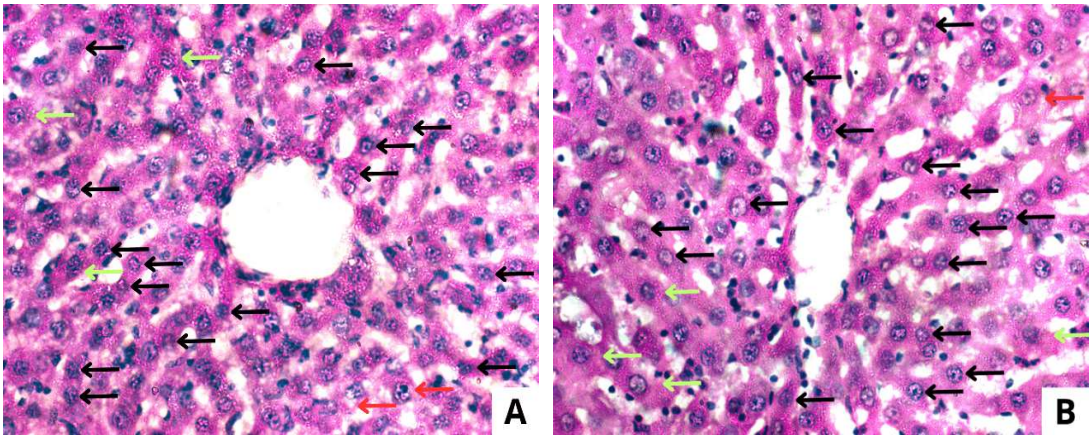

Figure 1. The histopathological features of liver (400X, HE staining). A: The control rat of the main test, B: The treatment rat of the main test. Black: normal, green: parenchymatous degeneration, red: hydropic degeneration.

Table 1. The average histopathological score per liver cell

Group	Mean ± SD
Control	1.226 ± 0.0065
Treatment	1.235 ± 0.0079

Discussion

The liver is a complex organ that plays crucial roles in the body's metabolism and detoxification processes (Taek et al., 2019). Histopathological examination can reveal structural changes in the liver during safety test. For these examinations, the right lobe of the rat liver is typically used. This lobe is anatomically more vascularized than the others, featuring the dorsal branch of the right lateral lobe, the intermediate branch of the right lateral lobe, and the ventral branch of the right lateral lobe (Vdoviaková et al., 2016). Previous studies investigating the hepatotoxic effects of various substances have focused on the right lobe, such as research that observed liver cell damage caused by organophosphate induction (Jalili et al., 2019). Another study found that sodium arsenide induction led to more severe liver cell damage in the right lobe compared to the other lobes (Adeluwoye et al., 2017).

Observation of the histopathological liver damage was around the central vein. Liver cells in zone 3 have distinct functions compared to those in other zones, as they contain the highest concentrations of cytochrome P-450 enzymes, which are responsible for metabolizing various substances. Consequently, liver cells in zone 3 are most significantly affected by exposure to exogenous substances, making histopathological changes in these cells most apparent in this zone (Maulina, 2018). Qualitative analysis revealed the presence of parenchymal degeneration and hydropic degeneration in the similiar degree, while necrosis was absent in both groups. These findings showed that the degeneration observed weren't caused by SPE. Parenchymal degeneration is linked to ATP depletion caused by exposure to foreign substances, which disrupts the transport of proteins synthesized by ribosomes out of the hepatocytes. The subsequent stage of damage is hydropic degeneration, which is also related to ATP depletion. This condition leads to an influx of water and sodium ions into the cell, while potassium ions are expelled. As a result, the cells are unable to remove excess water and organelles, leading to water accumulation within the cells (Sari et al., 2018).

In this study, OECD TG 420 was selected to evaluate the acute oral toxicity of the test compound, aligning with the study's primary objective to determine the substance's potential toxic effects following a single exposure and to establish its hazard classification. The use of OECD TG 420 is appropriate at this preliminary stage, as the goal is not to assess chronic or cumulative toxicity, but rather to identify observable signs of toxicity and estimate the dose range associated with adverse effects. The results of the statistical analysis of the liver histopathological scores between the control and treatment groups indicated no significant difference ($p>0.05$). This approach is consistent with the study conducted by Chaurasiya et al., 2025 who applied the OECD TG 420 guideline to assess the acute oral toxicity of Mangifera indica Linn. leaf extract at a dose of 2000 mg/kg body weight. In that study, histopathological evaluation of liver tissues using hematoxylin and eosin (H&E) staining revealed no signs of mortality or significant morphological alterations. The livers of both treated and untreated mice displayed normal hepatocyte architecture and intact structures of the central vein and sinusoids, comparable to the control group. In addition, in another organ, (Abrori et al., 2019) conducted a study on the acute toxicity test of ethanol extract of basil leaves. The study used the OECD TG 420 method with a dose of 2,000 mg/kg BW. The results showed that at a dose of 2,000 mg/kg body weight, the compound was classified as non-toxic; however, there were changes in the histopathological appearance of the mice's kidneys in the form of focal lesions.

However, further examination of liver function through the assessment of liver enzyme levels, specifically alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and bilirubin, is necessary. Additionally, identifying liver inflammation or oxidative stress using immunohistochemistry methods, as well as observing other microscopic liver structures such as sinusoidal congestion and portal tract inflammation, would be valuable in addressing the limitations of the study (Moustafa & Ali, 2021). The toxicity test could also be conducted using alternative methods, such as in vitro cytotoxicity assays (ASEAN, 2014). This study only evaluated the effects of a single dose of SPE, so further toxicity studies using repeated doses administered over a period of 28 days (OECD TG 407) or 90 days (OECD TG 408) are still needed.

Conclusion

Based on the result of this study, it can be concluded that SPE 5,000 mg/kg does not show acute toxic effects in rats, with LD_{50} estimated at >5,000 mg/kg according to OECD standards.

Conflict of Interest

The authors state no conflict of interest.

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Author contribution

RD & DH conceived the planned experiments, NAP conducted the experiments, NAP, RD, DH, & DA contributed in the data interpretation and analysis, SR provided the critical feedback, and all author contributed in the manuscript writing with RD as the lead.

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