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Acute Toxicity Test of Shallot (*Allium cepa* L.) Peel Extract on Ovarian Follicles Count in Rats (*Rattus norvegicus*)

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ABSTRACT: Shallot skin (*Allium cepa L.*) is a natural material with potential as a traditional medicine due to its high content of the antioxidant quercetin. However, excessive consumption of shallot skin may cause the antioxidant properties of quercetin to shift to pro-oxidant effects via auto-oxidation and metal-binding reactions. Uncontrolled pro-oxidants induce oxidative stress that damages body cells, including ovarian follicle cells. This acute toxicity study aimed to evaluate the toxic effects of shallot skin extract on the ovary using ovarian follicle count as an indicator. This study employed a true experimental design with a posttest-only control group. The method referred to the OECD 420 guideline (fixed dose procedure) using rats (*Rattus norvegicus*) as test animals. The results showed no significant difference in the mean follicle counts between the control and treatment groups across all follicle categories: primary follicles ($p = 0.278$), secondary follicles ($p = 0.452$), DeGraaf follicles ($p = 0.39$), corpus luteum ($p = 0.752$), atretic follicles ($p = 1.0$), and total follicles ($p = 0.60$). Thus, follicle counts in all categories did not differ significantly ($p>0.05$). It can be concluded that shallot skin extract does not exert toxic effects on ovarian follicle numbers in rats.

Keywords: Shallot skin; ovary; follicle; acute toxicity test

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INTRODUCTION

Shallot (*Allium cepa L.*) is a horticultural crop widely recognized for its health benefits. It has demonstrated positive effects in reducing or inhibiting inflammation, microbial infections, diabetes, and cancer. These beneficial effects are attributed to various active compounds or phytochemicals present in shallots. The predominant phytochemical found in shallots is the flavonoid-type antioxidant, particularly quercetin (Shahidi & Ambigaipalan, 2015). Notably, the outermost layer of the shallot bulb—commonly discarded as waste—contains up to 80% higher quercetin content compared to the bulb itself (Dibal et al., 2020)

In the human body, quercetin can neutralize oxidants and free radicals by donating electrons or hydrogen atoms. However, numerous studies have indicated that high doses of quercetin can alter its function from antioxidant to pro-oxidant (Kocyigit & Selek, 2016). This shift is due to the increased formation of quercetin-derived radical products, such as semiquinone and o-quinone. Although these radicals are relatively less reactive, they are unstable and possess potential cytotoxicity to cells. Furthermore, the metal-chelating property of quercetin can enhance the Fenton reaction, thereby increasing the production of highly reactive hydroxyl radicals (Papuc et al., 2017).

Dosage is a critical determinant in whether quercetin acts as an antioxidant or a pro-oxidant (Chen et al., 2014). Toxicity testing of shallot peel extract using the Brine Shrimp Lethality Test (BSLT) demonstrated that the extract falls under the category of moderate toxicity, showing 50% lethality to Artemia salina larvae. The study also revealed a dose-dependent relationship, where higher extract concentrations resulted in increased larval mortality (Fitriyanti et al., 2024). Nonetheless, the results of the BSLT cannot directly predict toxic effects in mammals. A study by Dibal (2020) reported nephrotoxic effects of quercetin—the principal compound in shallot peels—on rats administered a daily dose of 380 mg/kg body weight for 28 days. Histopathological examinations revealed tubular degeneration and glomerular distortion. Therefore, further in vivo toxicological assessments of shallot peel extract in mammals are warranted, beginning with acute toxicity tests based on international guidelines.

The most widely recognized framework is that of the Organization for Economic Co-operation and Development (OECD). The OECD Test Guideline 420 is specifically designed for acute toxicity testing in rodents and emphasizes observation of toxicological signs using a fixed-dose procedure. Comprehensive organ examinations are conducted under OECD guidelines, including the reproductive system (OECD, 2001). As previously discussed, high doses of quercetin may exert pro-oxidant effects that can lead to cellular cytotoxicity.

Uncontrolled pro-oxidant activity contributes to oxidative stress, which may impact ovarian function. Additionally, the quercetin contained in shallot peel extract exhibits estrogen-like activities and is sensitive to estrogen receptors in the body (Slighoua et al., 2023). Estrogen and its receptors play a critical role in maintaining granulosa cell and oocyte development in the ovaries (Tang et al., 2019). Therefore, an acute toxicity study focusing on the ovaries—using the number of ovarian follicles as a key indicator—is essential to evaluate the safety of shallot peel as a traditional medicinal ingredient. Moreover, toxicity testing is a vital step in the drug development process to assess potential adverse effects on human health, beginning with acute toxicity evaluations (BPOM, 2022).

METHODS

This study employed a true experimental design using a post-test only control group design. The method employed refers to the OECD Guideline 420. Ethical approval for this research was obtained from the ethics committee of Faculty of Medicine, University of Jember, with the clearance number 1637/UN25.1.10.2/KE/2024.

Preparation of Shallot Peel Extract

The extraction process began with the preparation of dried shallot peel material (simplicia). Shallot peels were obtained from Tanjung Market in Jember and subsequently sorted to remove residual bulbs and impurities. To eliminate pesticide residues, the peels were soaked in 2% saline solution and rinsed under running water. The cleaned peels were then sun-dried and ground into powder using a blender. The resulting simplicia was subjected to maceration using 96% ethanol as a solvent at a 1:10 (w/v) ratio. The mixture was allowed to stand for three days with intermittent stirring once daily. The extract was filtered using Whatman No. 2 filter paper. The filtrate was concentrated using a rotary evaporator until all solvent was evaporated. The semi-solid extract was then dried in an oven at 50°C to obtain the final concentrated extract.

Acute Toxicity Testing

Acute toxicity testing in this study refers to the OECD guideline 420 (fixed dose procedure) with the objective of determining the lowest dose of red onion skin extract that can cause toxicity. The test subjects were rodents (*Rattus norvegicus*) in accordance with the principles of replacement, reduction, and refinement (3Rs). The procedure consisted of three tests: preliminary test, main test, and limit test. The preliminary test used one rat with dose options of 5, 50, 300, and 2,000 mg/kg body weight (BW). Based on the study by (Putri et al., 2020) which estimated the LD50 of red onion skin extract to be >2,000 mg/kg BW, the preliminary test in this study started at a dose of 2,000 mg/kg BW. The extract was administered as a single dose, followed by observations for signs of toxicity (behavior, physical condition, somatomotor activity, autonomic nervous system, central nervous system, and respiratory system) every 30 minutes, then every 4 hours for 24 hours, and once daily for 14 days. If no signs of toxicity were observed, the preliminary test was continued with a dose of 5,000 mg/kg BW. The main test was conducted using doses determined from the preliminary test results, while the limit test was performed if the LD50 dose was >2,000 mg/kg BW.

Animal Treatment Protocol

The test animals were acclimated to laboratory conditions for 7 days. The preliminary test was conducted by administering a single dose of red onion skin extract at 2,000 mg/kg body weight to rats that had been fasted overnight. Following the administration of the extract, the rats were observed for clinical signs of toxicity during the first 24 hours, and no signs were detected. Therefore, the preliminary test was continued with a higher dose of 5,000 mg/kg body weight. Subsequently, the main test was performed using a control group (3% DMSO solution) and a treatment group (shallot skin extract at 5,000 mg/kg body weight), with 5 rats in each group. The volume of DMSO solution and extract administered was calculated based on the gastric capacity of the rats, at 1 ml per 100 grams of body weight. Toxicity signs were observed over a 14-day period. A separate limit test was not necessary because the preliminary test with a dose of 5,000 mg/kg body weight followed by the main test with the same dose fulfilled the requirements of the limit test procedure.

Histological Preparation and Data Analysis

On day 15, the animals were euthanized for organ collection. Euthanasia was performed using ketamine-xylazine anesthesia followed by cervical dislocation. The

animals were fixed on dissection boards, and the ovaries were collected. The carcasses were buried at a depth of 50 cm in accordance with ethical disposal procedures.
Ovarian tissues were processed using the paraffin embedding method and stained with hematoxylin and eosin (H&E). Histological analysis was conducted using an Olympus BX53 light microscope. Two researchers independently evaluated all fields of view at 40× magnification, counting the number of primary follicles, secondary follicles, Graafian follicles, atretic follicles, corpora lutea, and total follicles in both control and treatment groups. Data were analyzed using appropriate statistical methods, specifically Independent T-tests and the Mann–Whitney U test, depending on the distribution characteristics of the data.

RESULT AND DISCUSSION

This acute toxicity study utilized the number of ovarian follicles as the dependent variable. Follicle classification was based on the criteria defined in DiFiore Histology Atlas (Eroschenko, 2015), which categorizes follicles into five types: primary follicles, secondary follicles, Graafian follicles, corpora lutea, and atretic follicles. The follicle count data for both control and treatment groups are presented in Table 1.

Table 1. Follicle Count Data of Control and Treatment Groups

	Mean ± Standard Deviation	
Follicle	**Control Group**	**Treatment Group**
Primary Follicle	3.0 ± 1.5	2.0 ± 1.4
Secondary Follicle	3.6 ± 2.8	2.0 ± 1.0
Graafian Follicle	0.6 ± 0.8	1.4 ± 1.3
Corpus Luteum	5.6 ± 4.3	5.0 ± 1.8
Atretic Follicle	0.2 ± 0.4	0.2 ± 0.4
Total Follicle	12.8 ± 8.0	10.4 ± 3.9

The data from both groups showed a dominance of corpora lutea compared to other follicle types, with a mean corpus luteum count of 5.6 in the control group and 5.0 in the treatment group. The follicle type with the lowest mean count was the atretic follicle, with both groups exhibiting the same mean value of 0.2.

The data analysis using the Mann-Whitney test revealed p-values greater than 0.05 ($p > 0.05$) for all follicle categories (Table 2). This indicates that the null hypothesis cannot be rejected, meaning there is no significant difference in follicle counts between the control and treatment groups.

Table 2. Statistical Test for Comparison Between Control and Treatment Groups

	Mann-Whitney Test					
	Primary Follicle	**Secondary Follicle**	***Graafian Follicle***	**Corpus Luteum**	**Atretic Follicle**	**Total Follicle**
Asymp. Sig. (2-tailed)	0.278	0.452	0.309	0.752	1.000	0.600

A study done previously (Cahya et al., 2022) demonstrated the hepatoprotective effects of ethanol extract of shallot skin in rats induced with diazinon. Additionally, the ethanol extract of shallot skin showed gastroprotective effects in male Wistar rats administered mefenamic acid (Putri et al., 2020). These beneficial effects are attributed to the flavonoids present in shallot skin. The main flavonoid contained in shallot skin is quercetin(Shahidi & Ambigaipalan, 2015). Quercetin possesses antioxidant, antibacterial, anti-inflammatory properties, and protective effects on various organs, including the ovary. Another study investigated female Wistar rats exposed to cadmium chloride to induce reproductive toxicity, finding that quercetin administration before, during, and after cadmium exposure reduced cadmium toxicity, as evidenced by decreased follicular cell apoptosis and increased serum reproductive hormone levels. Research by (Beazley & Nurminskaya, 2016) revealed positive effects of quercetin on ovarian folliculogenesis, histopathologically indicated by an increased number of antral follicles in rats treated with quercetin at a dose of 5 mg/kg/day continuously for nine months.

The effects of quercetin on the ovary are due to its estrogen-like activities, rendering it sensitive to estrogen receptors in the body (Slighoua et al., 2023). Estrogen receptors mediate the effects of estrogenic compounds on target tissues, and in the ovary, these receptors play a crucial role in the growth and development of granulosa cells, oocytes, and overall ovarian function (Tang et al., 2019).

However, several studies have indicated that high doses of quercetin can shift its beneficial antioxidant properties to pro-oxidant effects (Kocyigit & Selek, 2016). Quercetin can neutralize free radicals by donating electrons or hydrogen atoms; however, this reaction produces quercetin-derived radical species with low reactivity (quercetin semiquinone and o-quinone). Although these compounds have low reactivity, they are unstable and potentially cytotoxic to body cells (Papuc et al., 2017). At low concentrations and under normal antioxidant-prooxidant cellular balance, these products are rapidly neutralized by the body's glutathione (GSH) enzymes. Problems arise when quercetin concentrations exceed available GSH enzymes, allowing pro-oxidant effects to dominate and potentially induce oxidative stress (Yang et al., 2020). Dibal's study in 2020 demonstrated nephrotoxic effects in rats administered quercetin at a dose of 380 mg/kg body weight daily for 28 days, with histopathological evidence of tubular degeneration and glomerular distortion(Dibal et al., 2020). A research in 2024 reported that high doses of quercetin (2,000 mg/kg body weight) caused cytotoxic effects, evidenced by increased biomarkers such as aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and alanine aminotransferase (ALT), leading to quercetin being described as a versatile antioxidant or "double-edged sword" (Mishra et al., 2020). Besides excessive dosing, quercetin's pro-oxidant property in shallot skin extract may also stem from its strong metal ion chelating characteristic. Quercetin's ion-chelating activity enhances Fenton reactions, increasing hydroxyl radical formation in the body. This disrupts the homeostatic balance between antioxidants and pro-oxidants (Thao et al., 2019). Therefore, toxicity testing of shallot skin extract is necessary. As an initial step, this study analyzed the acute toxicity through histopathological assessment (follicle count) of the ovary.

In this study, administration of shallot skin extract at a single dose of 5,000 mg/kg body weight did not result in significant differences between the control and treatment groups. Histopathological examination of both groups revealed a dominance of corpora lutea with minimal follicular atresia (Figure 1). The corpus luteum is a temporary endocrine gland formed following follicular rupture during ovulation. The dominance of corpora lutea in both groups indicates that the ovaries were in the metestrus-diestrus

phase at the time of termination. The metestrus and diestrus phases in rats are homologous to the luteal phase in humans (Ajayi & Akhigbe, 2020). This phase is characterized by hypertrophy and differentiation of follicular cells (granulosa and theca cells), followed by rapid angiogenesis in the granulosa cell region (Taketa, 2022). This process results in corpus luteum morphology featuring a collapsed capsule with folds, and a central region containing blood vessels and loose connective tissue (Eroschenko, 2015). The luteal phase is part of the estrous cycle, which represents the ovarian cycle pattern marking the receptive and non-receptive reproductive phases in mammals, thereby allowing for possible pregnancy (Crowe, 2022). The uniformity of the estrous cycle phases across all test animals was attributed to identical environmental conditions. Temperature, photoperiod, and food supply are among the extrinsic factors influencing the estrous cycle in animals. Additionally, all test animals were of similar age, which is an intrinsic factor affecting the estrous cycle (Alfiyanti et al., 2019).

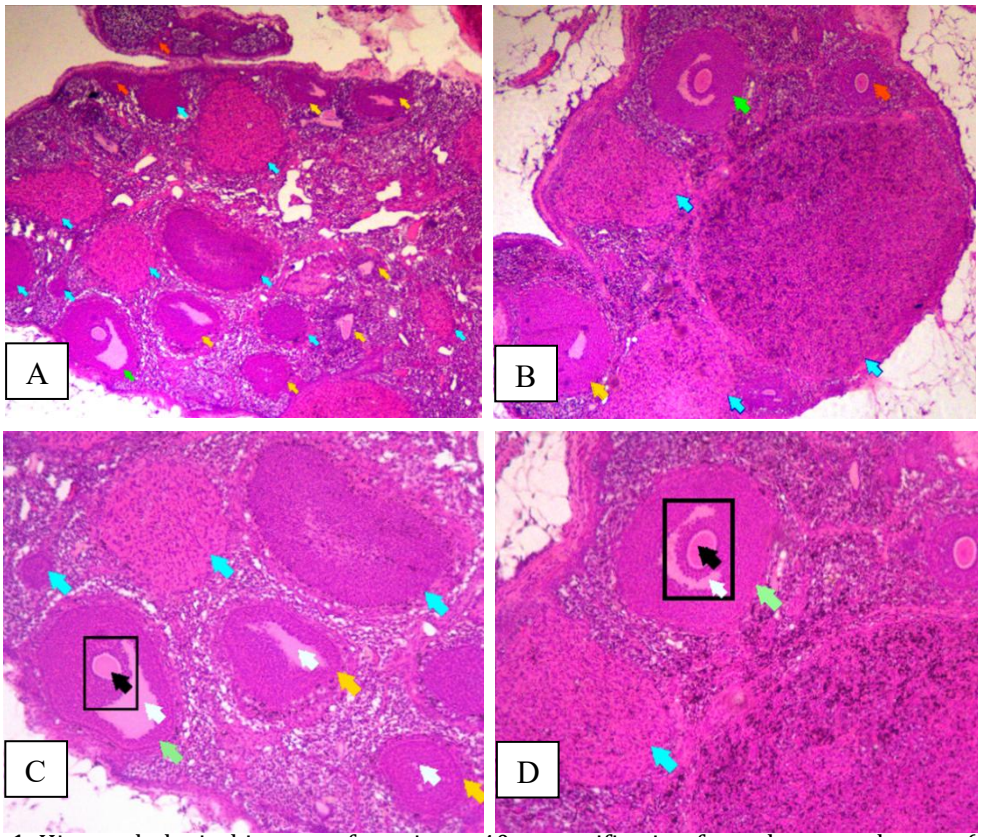

Figure 1. Histopathological images of ovaries at 40× magnification from the control group (a) and treatment group (b), and at 100× magnification from the control group (c) and treatment group (d). Staining was performed using hematoxylin and eosin (H&E). Images were captured with an Olympus BX53 microscope and analyzed using Optilab Viewer 2.2. Red arrows indicate primary follicles, yellow arrows indicate secondary follicles, green arrows indicate Graafian follicles, and blue arrows indicate corpora lutea. In the Graafian follicle, the oocyte morphology is shown by the black arrow, surrounded by the corona radiata (black box), with the antral cavity visible nearby (white arrow).

In this study, administration of a single dose of 5,000 mg/kg body weight of shallot skin extract using the OECD 420 acute toxicity method in rats did not show any toxic effects either clinically or histopathologically. Therefore, it can be concluded that shallot skin

extract is classified as non-toxic to the ovary. Contrasting results were reported in a toxicity study using the BSLT method by Fitriyani and Elsyana (Fitriyanti et al., 2024). Their study found that shallot skin extract exhibited toxic effects on shrimp larvae, classifying it as moderately toxic. The discrepancy between these two studies is attributed to differences in methodology and test organisms used. In shrimp larvae, phytochemicals in the shallot skin extract acted as antifeedants and were toxic to the larval stomach, leading to larval mortality (Elsyana & Tutik, 2018). Such toxic effects were not observed in rats administered 5,000 mg/kg body weight of shallot skin extract in the present study.

This study has several limitations. Regarding natural substances, there is a divergence of expert opinions on the necessity of toxicity testing. The ASEAN Botanical Safety Assessment guidelines state that if a natural substance is commonly consumed by the public, a local tolerance assessment is sufficient, and toxicological testing may not be required. Shallot falls under this category. In the community, shallot is widely used as a cooking ingredient, and no reports of adverse effects from its consumption have been documented. Therefore, referring to these guidelines, it is recommended that future studies employ alternative toxicity testing methods, such as in vitro cytotoxicity assays (cell-based cytotoxicity) or computer-simulated toxicity models. These methods allow direct observation of interactions between specific proteins or phytochemicals in shallot skin extract and target cells.

CONCLUSION

Based on the acute toxicity test of shallot skin extract (*Allium cepa L.*) in this study, it can be concluded that a single dose of 5,000 mg/kg body weight of shallot skin extract does not cause toxic effects on the ovarian follicle count in rats (*Rattus norvegicus*).

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AUTHOR CONTRIBUTION

DH: Concepts or ideas; design; definition of intellectual content
ND: Literature search; experimental studies; data analysis; manuscript preparation.
MH: Definition of intellectual content; manuscript review.
RS, SR: Experimental studies; manuscript editing; manuscript review.
AM: Ethical proposal; literature search.

ETHICS APPROVAL

The in vivo test had been approved by the ethics commission of Faculty of Medicine Universitas Jember with number of 1637/UN25.1.10.2/KE/2024.

CONFLICT OF INTEREST

None to declare

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