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RESEARCH ARTICLE

Acute Toxicity of 70% Ethanol Extract of *Kenitu* (*Chrysophyllum cainito* L.) Leaves of Wistar Rats (*Rattus norvegicus*)

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ABSTRACT

The postmenopausal phase, which results in a lack of estrogen levels and is one of the triggers of menopausal symptoms, contributes to the elderly's cognitive function decline. This condition can also cause joint and bone pain, bladder and urinary tract disorders, and sexual problems. Based on several studies, it is known that *kenitu* (*Chrysophyllum cainito* L.) contains phytoestrogen compounds with a structure similar to the hormone estrogen, so *kenitu* has the potential as an alternative treatment for disease conditions with risk factors for estrogen deficiency. The study was conducted to determine the estimated lethal dose 50 (LD_{50}) value based on the Globally Harmonized System (GHS) classification as well as macroscopic (physical, behavioral, and average weight gain per day) and microscopic (liver and kidney histology) values in Wistar rats against the administration of 70% ethanol extract of *kenitu* leaves at a dose of 2000 mg/kg body weight (BW). The Up-and-Down Procedure (UDP) acute toxicity test method was used for this study. The maximum dose was 2000 mg/kg BW of ethanol extract from 70% kenitu leaves. The study found that the 70% ethanol extract of *kenitu* leaves had more than 2000 mg/kg BW LD_{50} value against Wistar rats. Based on the classification of the GHS, it can be concluded that 70% ethanol extract of *kenitu* leaves against Wistar rats is classified as low toxicity.

Keywords: Acute toxicity, *Chrysophyllum cainito* L., estrogen deficiency, OECD425

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Introduction

Postmenopausal women generally have ovaries that are smaller in size and do not contain follicles. About 3–4 years before reaching menopause, there is an increase in follicle-stimulating hormone (FSH) levels, and the production of estrogen, inhibin, and progesterone by the ovaries decreases. The menopausal process changes due to the reduced number of follicles and the interaction between the central nervous system and the ovaries [1]. This decrease in the number of follicles will continue, leading to a decrease in the stimulation of estrogen hormone production. As a result, estrogen hormone levels decrease [2].

According to other studies, a decrease in estrogen levels can lead to a variety of symptoms, including joint and bone discomfort, physical and mental exhaustion, bladder and urinary tract issues (difficulty urinating, uncontrolled urination), sexual problems (changes in sexual desire, sexual activity, and sexual satisfaction) and complaints of vaginal dryness (vaginal dryness or burning and difficulty having sex) [1].

The most optimal method of coping with the signs of menopause is to restore the level of the hormone estrogen, since almost all manifestations that appear during the postmenopausal phase come from a decrease in estrogen levels. Hormone replacement therapy (HRT) is the therapeutic strategy that restores this hormone. Research consistently and effectively reduces menopausal symptoms like hot flushes, vaginal atrophy, insomnia, bone loss, and fracture risk, while also preventing cardiovascular disease [3]. Although HRT has its advantages, keep in mind that there is a risk of certain side effects. Long-term observations of women undergoing HRT show an increased risk of breast, endometrial, ovarian, and thromboembolism [3], [4]. Because of these risks, it is necessary to have alternative treatment methods with an effectiveness level equivalent to HRT but safer. In this context, the utilization of natural components from plants containing phytoestrogens is considered a relevant alternative [4], [5].

Kenitu (*Chrysophyllum cainito* L.) is a plant commonly found in downstream areas to valleys. In previous research, the 96% ethanol extract derived from *kenitu* demonstrated efficacy in augmenting the osteoblast cell count within the trabecular vertebrae of male mice induced with dexamethasone [6]. The leaves of *kenitu* plants have been studied before and found to contain flavonoids, phenols, gentisic acid, *β*-amirin acetate, *β*-sitosterol, phenols, gallic acid, lupeol, ursolic acid, sterols, and triterpenoids [7]. There are flavonoid groups, like apigenin, 6-genistein, kaempferol, daidzein, catechins, and quercetin compounds, that can make plants produce estrogen [5]. The class of phytoestrogen compounds found first in plants that have a shape resembling the hormone estrogen, so it has the potential as an alternative medicine or herbal medicine for diseases with risk factors for estrogen deficiency [7].

Herbal treatment must meet quality standards set by the World Health Organization (WHO). The standard covers aspects of safety, quality, and efficacy. A series of preclinical experiments, including toxicity tests, can test the safety of

herbal medicines. Acute toxicity testing is the first step in toxicity testing. Before its use, acute toxicity testing data are required for clinical trial evaluation and drug safety. The acute toxicity test results play a crucial role and serve as the initial requirement for conducting clinical trials. Acute toxicity tests were used to determine the dose that could cause death in 50% of the test animals. Furthermore, acute toxicity tests establish the dosage range for subsequent tests, like sub-chronic toxicity tests, and aid in identifying poisoning issues [8]. Based on the previous explanation of the benefits of *kenitu* leaves as an alternative treatment option for conditions associated with lack of estrogen, it is necessary to conduct a toxicity test of 70% ethanol extract of *kenitu* leaves in Wistar rats to determine the level of safety, which is assessed through estimated LD_{50} values as well as macroscopic (including physical, behavioral, and average weight gain per day) and microscopic (liver and kidney histology) observations.

Materials and Methods

Materials

1. Plants

Kenitu (*Chrysophyllum cainito* L.) leaves were collected in Batu, Indonesia, in June 2022 and identified in UPT. Laboratorium Herbal Materia Medica, Batu, Indonesia with identification letter No. 067/1606/102.20/2023. The leaves were prepared to get dry powder of *kenitu* leaves.

2. Animals

Wistar rats (*Rattus norvegicus*) was obtained from the experimental animal laboratory of the BioSains Institute, Universitas Brawijaya, Malang, Indonesia, with ethical clearance letter No. 136-KEP-UB-2023.

3. Chemicals

The chemicals used were 70% ethanol, 1% tween 80, 0.5% DMSO, 96% alcohol, 80% alcohol, 0.9% sodium chloride (NaCl), 2% sodium sulfate, 10% formalin solution, aluminum chloride, hydrochloric acid, paraffin solution, 1% acid alcohol, hematoxylin dye, aqueous ammonia, formic acid, 1% eosin comparison paint, and xylene were purchased from Merck® (Darmsadt, Germany).

Methods

1. Acute Toxicity Test

Before treatment, female Wistar rats underwent 18-hours fasting period. In this phase, rats were allowed to drink water but not allowed to be fed. Furthermore, rats were weighed and given a test substance in the form of a single dose of 2000 mg/kg BW, with the volume of administration adjusted to the rat's body weight [9].

One rat was treated by giving 70% ethanol extract of *kenitu* leaves at a single dose of 2000 mg/kg BW through oral administration according to the rat's body weight. Following treatment, one rat was fasted for four hours. If the rat survived for 48 hours post-treatment, four other rats would be administered the same dose. However, in the event of mortality within the first 48 hours, the main test would be initiated [10].

After observation for 48 hours, if the first rat survive, four additional rats would receive the same oral dose (2000 mg/kg BW) of the 70% ethanol extract of *kenitu* leaves. Furthermore, the four replicated rats would also be fasted for four hours. The Globally Harmonized System (GHS) can further classify the estimated LD_{50} value if the number of rats surviving until observation on day 14 is less than three. However, the next stage of testing, known as the main test, begins when the number of rats that cannot survive or die reaches three or more [10].

2. Changes in Rat's Behavior

Observations on rats were made at the 0 minute before administration, and then at 30, 60, and 120 minutes after administering the 70% ethanol extract of *kenitu* leaves. The observation process was carried out repeatedly in the first 24 hours and continued for 14 days [11]. Measurement of several symptoms of the emergence of toxicity responses, which include skin and fur conditions, eye changes, lethargy, breathing, trembling or tremors, salivation, diarrhea, and death [9], [11]. Macroscopic observations were made by at least three people to evaluate signs of toxicity by comparing changes in control group and the treatment group of 70% ethanol extract of *kenitu* leaves [12], [13].

3. LD_{50} Calculation

Both short-term (48 hours) and long-term (14 days) rat mortality were associated with the estimated LD_{50} value. The LD_{50} was calculated using the Probit Analysis software. Data related to dose and response from all test animals were entered into AOT425StatPgm to determine the estimated LD_{50} value.

4. Average Weight Gain Per Day of Rats

Weighing on rats was carried out before, momentarily, and after administering the 70% ethanol extract of *kenitu* leaves. Then the average weight gain per day of rats would be recorded and analyzed statistically using SPSS software [13].

5. Data Analysis

Data analysis was carried out by calculating the average of each treatment group, which aims to determine and compare weight changes every day of the control group and the grup receiving 70% ethanol extract of *kenitu* leaves. The results of the research data were tested using the T-test with a confidence level of 95% at $p > 0.05$.

6. Hepatic and Renal Histology

On the 15th day, the test animals were euthanized by dislocating neck method and underwent surgery to collect liver and kidney samples [14]. Histological preparations were made using the Hematoxylin-Eosin (HE) staining method, which includes washing, dehydration, cleaning, infiltration, insertion, slicing, staining, and installation stages. Furthermore, the preparation was observed under a light microscope with the help of an Optilab microscope camera connected to a computer using a magnification of 400x at 5 fields of view. Microscopic organ observation includes fat degeneration, hydropic degeneration and inflammation.

Result

LD_{50} Value Estimation Based on GHS

Data derived from acute toxicity testing using the OECD425 methods were then inputted to the AOT425StatPgm software developed by the US Environmental Protection Agency. The calculation of the estimated LD_{50} value was

carried out based on the dose that caused death in the test animal [14]. The LD_{50} value of 70% ethanol extract of *kenitu* leaves in Wistar rats was estimated through AOT425StatPgm software showing LD_{50} results > 2000 mg/kg BW.

Average Weight Gain Per Day of Rats

Figure 1 illustrates weight fluctuations in both the control and 2000 mg/kg BW dose groups. The control group exhibits variations in average weight gain/loss on day 7 (acclimatization), days 5-7 and day 10 (post-treatment). Similarly, the treatment group shows weight fluctuations on day 1-4 and day 7 (acclimatization), day of treatment (minute 30), days 1-4, day 7, day 1-2, and day 8 (post-treatment).

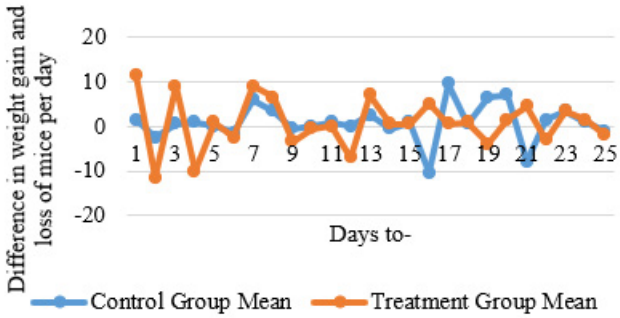
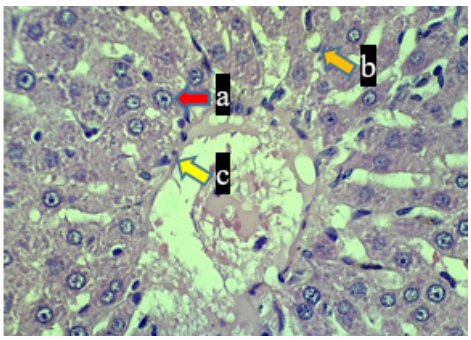


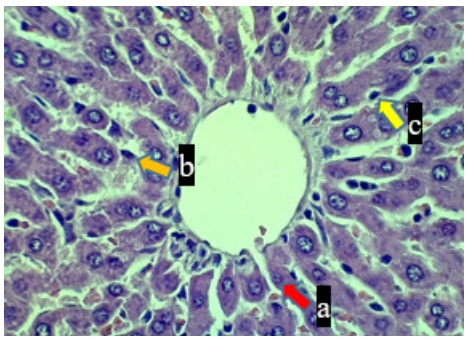
Figure 1. Graph of average weight gain per day of Wistar rats control group and dose 2000 mg/kg BW

Hepatic and Renal Histology

Figures 2 show observations of the liver and kidneys of Wistar rats given a 70% ethanol extract of *kenitu* leaves at doses of 2000 mg/kg BW and given aquadest as a control. Both the control and treatment group showed similar features of mild liver and kidney morphological damage. This is clear that liver damage is seen as affecting 1/3 to 2/3 of the liver cells' field of view and kidney damage is seen as affecting 51 to 75% of kidney cells through cell death and degeneration.



(A)



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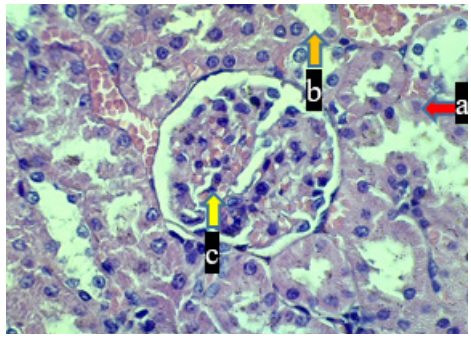
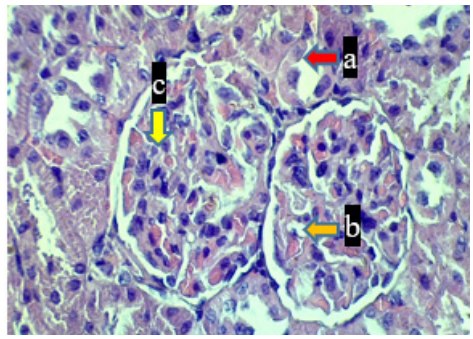


(C)



(D)

Figure 2. The liver cells in the control group were given aquadest (A), the liver cells in the treatment group were given a 70% ethanol extract of *kenitu* leaves at a dose of 2000 mg/kg BW (B), the renal tubule epithelial cells in the control group received aquadest (C), while the renal tubule epithelial cells in the treatment group received a 70% ethanol extract of *kenitu* leaves at a dose of 2000 mg/kg BW (D). This resulted in (a) hydropic degeneration, (b) fat degeneration, and (c) inflammatory responses

Discussion

The Up-and-Down Procedure (UDP) is the simplest method for determining the estimated LD_{50} value. In addition, the Handbook on Non-Safety Clinical Testing states that WHO recommends the use of the UDP method in acute toxicity testing. This recommendation is based on the nature of flexibility, as well as the economic, scientific, and ethical [14].

Previous studies have indicated that the test material may not be toxic, leading to the use of the limit test stage in the UDP. Understanding similar test compounds, which take into account the identity and percentage of components known to have toxicological significance, can provide information about the toxicity level of test materials. According to the limit test method guide, the determined dose ranges from 2000 to 5000 mg/kg BW. It is recommended to use a dose of 2000 mg/kg BW because testing at a dose of 5000 mg/kg BW is only considered when there is strong evidence that the test results will be directly beneficial to protect animal or human health [9], [15].

In this study, the LD_{50} was determined by observing and counting mortalities throughout the experiment. Observations started from 0 minute before administration, and continued at 30, 60, and 120 minutes after administering a dose of 2000 mg/kg BW of 70% ethanol extract of *kenitu* leaves. The observation process was carried out repeatedly in the first 24 hours and continued for 14 days if there were no deaths [11]. No deaths occurred during observation, including during the observation period starting from 0 minute before administration, until the

first 24 hours after administration, as well as at every 24-hour interval for the following 14 days.

The LD_{50} value of 70% ethanol extract of *kenitu* leaves in Wistar rats was estimated through AOT425StatPgm software showing LD_{50} results > 2000 mg/kg BW. The GHS classification classifies preparations with an estimated LD_{50} value > 2000 mg/kg BW as low toxicity, meaning they do not cause toxicity symptoms in test animals and do not require additional safety warning symbols or signs on the label [9], [14], [16].

Apart from monitoring the number of deaths of experimental animals, the test also observes the symptoms of toxicity that appear before administration, as well as at 30, 60, and 120 minutes after administering the 70% ethanol extract of *kenitu* leaves. This observation was repeated at intervals within the first 24 hours after administration and continued for 14 days if there were no incidents of death [11]. Symptoms of toxicity observed include skin and coat conditions, eye changes, lethargy, breathing, shaking or tremors, drooling, diarrhea, and death [9], [12]. During observation of toxicity symptoms after administration of the lowest and single dose of 70% ethanol extract of *kenitu* leaves at 2000 mg/kg BW in rats, no significant signs of toxicity were detected. Treated rat groups showed similar activity to rats in the control group.

As shown in **Figure 1**, there was no significant difference (p-value > 0.05) in the average daily weight gain between negative controls and those administered the 70% ethanol extract of *kenitu* leaves [17]. Due to the single administration of the test preparations, it is difficult to definitively determine the cause of weight gain or loss in the animals. Individual conditions of each test subjects may have played a role [18]. However, other studies observed that feed composition, which contributes to nutrient intake, can influence significant increases in body weight [19], [20], [21]. Meanwhile, weight loss in the control group may be due to stress, especially because before the acclimatization period, the rats had an uncontrolled diet [21].

Figure 2 shows observations of the liver and kidneys of Wistar rats given a 70% ethanol extract of *kenitu* leaves at doses of 2000 mg/kg BW and given aquadest as control. These results are in line with research conducted by Shailajan and Gurjaj [22]. The study revealed that *kenitu* leaves extract significantly reduced malondialdehyde levels in rat liver and kidney homogenates and increased metallothionein in liver and kidney homogenates [23].

Conclusion

Based on the estimated LD_{50} value of the GHS classification, *kenitu* leaves are included in low toxicity so they do not cause symptoms of toxicity. This is evidenced in macroscopic (physical, behavioral, and average weight gain per day) and microscopic (liver and kidney histology) observations in Wistar rats against the administration of *kenitu* leaves dose of 2000 mg/kg BW.

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