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Ethanol extract of cassava (*Manihot esculenta* Crantz) peel is practically non-toxic after acute oral administration in male ddY mice

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ABSTRACT

Background: Cassava (*Manihot esculenta* Crantz) tuber peel contains active compounds with antidiabetic potential, but its safety has not been established.

Objective: To evaluate the acute oral toxicity of the ethanol extract of cassava tuber peel, determine its toxicity category, and estimate its LD_{50} in male ddY mice (*Mus musculus*).

Methods: Male ddY mice (n = 6 per group) received a single oral dose of the extract (1250, 2500, or 5000 mg/kg body weight [BW]) or 1% CMC-Na (control) according to BPOM Regulation No. 10 of 2022. Clinical signs, mortality, body weight, body temperature, and feed and water intake were observed for 14 days, followed by organ index determination.

Results: The extract contained flavonoids, saponins, and tannins. No mortality or clinical signs of toxicity occurred at any dose; only transient grooming was observed in all groups, including the control. Body weight, body temperature, feed and water intake, and organ indices did not differ significantly between groups ($p > 0.05$).

Conclusion: The LD_{50} of the ethanol extract of cassava tuber peel is greater than 5000 mg/kg BW, and the extract is classified as practically non-toxic after a single oral dose.

Keywords: *Manihot esculenta* Crantz, cassava tuber peel, acute oral toxicity, LD_{50}, ddY mice

Introduction

The use of natural ingredients in traditional medicine has been widely known among the Indonesian people since ancient times. As a country with high biodiversity, Indonesia holds great potential for the development of traditional medicines. The use of plants as natural remedies continues to grow, but it must be accompanied by attention to the safety aspects of their active compounds, especially for long-term use [1]. One such plant that contains active compounds with pharmacological potential is cassava (*Manihot esculenta* Crantz) [2].

Cassava is a plant from the Euphorbiaceae family that grows upright and produces starchy tubers, making it an important source of carbohydrates [3]. Commonly used parts of the plant include the tubers, leaves, and stems. However, cassava peel is often considered waste, particularly in staple food industries, agro-industries, and fermentation processes. Lampung Province is one of the largest cassava-producing regions in Indonesia. Nevertheless, cassava peel waste has yet to be optimally utilized by either industry or local communities. This peel waste accounts for approximately 16% of the total tuber weight and is still rarely exploited efficiently [4]. According to Statistics Indonesia (BPS), cassava is an important agricultural commodity in the region, with 314,775 agricultural business units recorded [5].

The peel of cassava tuber is known to contain secondary metabolites such as flavonoids, tannins,

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and saponins [6]. In addition, cassava peel contains cyanogenic glycosides, mainly linamarin and lotaustralin, which release toxic hydrogen cyanide (HCN) upon tissue disruption [7]. The content of cyanogenic compounds in the root cortex (peel) is generally higher than that in the root parenchyma (on average 804 vs. 305 ppm) [8], making the safety evaluation of peel-derived preparations particularly important. Various active compounds in cassava peel have been reported to exhibit pharmacological activities, including cholesterol-lowering, anti-obesity, antidiabetic, antioxidant, and antibacterial effects [9].

Although traditional medicines are generally perceived as safe due to their natural origin, this assumption does not necessarily reflect their actual safety. Previous studies have demonstrated the antidiabetic potential of cassava tuber peel extract [9]. However, since the treatment of high blood glucose levels is typically long-term, it is important to ensure that no toxic effects arise during use. Therefore, evaluating the safety level, particularly through toxicity testing, becomes a crucial step. Toxicity tests aim to identify potential harmful effects of a substance on biological systems. One such test is the acute oral toxicity test, which assesses adverse effects occurring after a single oral dose or multiple doses given within 24 hours [10].

These activities make cassava peel a promising candidate for development into standardized herbal medicines and phytopharmaceuticals. To support this development, a series of safety evaluations is required, including acute oral toxicity testing. This test is crucial as part of the new drug registration process to ensure the safety of use and to detect any potential toxic effects of cassava peel extract. In toxicological research and related fields such as pathology, histopathology, and genetics, mice are frequently used as test animals. Their selection is based on biological similarities to humans, including anatomical, physiological, reproductive, circulatory, and respiratory system characteristics [11,12]. However, to the best of our knowledge, the acute oral toxicity of the ethanol extract of cassava tuber peel has not been reported. Therefore, this study aimed to evaluate the acute oral toxicity of this extract in male ddY mice, determine its toxicity category, and estimate its LD_{50}.

Methods

Sample collection and botanical identification

Cassava tuber peel (*Manihot esculenta* Crantz) was obtained from a home-based industry (Singkong Keju Cap Sapi Perah), located in Bandar Lampung, and sourced from plantations in Purwodadi District, East Lampung Regency, Lampung Province. This region is situated at an altitude of 25–55 m above sea level. The plant material (cassava tuber peel) underwent botanical identification at the Botany Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), University of Lampung.

Preparation of simplicia and extraction

The test material used was the cream-colored epidermal peel of cassava tuber. The initial steps included wet sorting to remove dirt or foreign materials attached to the peel. The cassava peel was then sliced to increase surface area and sun-dried for approximately three days [13]. After drying, dry sorting was conducted to remove any remaining impurities. The resulting dried material (simplicia) was then ground using a grinder to obtain a fine powder ready for extraction.

The extraction process was carried out using the maceration method with 70% ethanol as the solvent, at a simplicia-to-solvent ratio of 1:10. A total of 250 g of simplicia powder was placed in a sealed jar, and maceration was conducted for three days (3 × 24 hours). The resulting macerate was filtered using a Buchner funnel, and the filtrate was concentrated using a rotary evaporator at 50 °C and 50 rpm, followed by further concentration using a water bath at 50 °C [14].

Phytochemical screening of simplicia and extract

Alkaloid test. One gram of simplicia powder and 1 g of cassava tuber peel extract were each mixed with ammonia solution and chloroform, then ground until homogeneous to initiate the basification process. The mixture was filtered, and 1 mL of 2 N HCl was added to the resulting filtrate, and the mixture was shaken vigorously. The organic phase was separated and divided into three test tubes. The first filtrate was treated with Mayer's reagent, the second with Dragendorff's reagent, while the third was left untreated as a control. A positive result was indicated by the formation of a white precipitate with Mayer's reagent and a reddish-brown or orange precipitate with Dragendorff's reagent [15].

Saponin test. Two grams of simplicia powder and two grams of extract were dissolved in 10 mL of water in a test tube, then vigorously shaken for 10 minutes. The formation of stable foam that persisted for 10 minutes after the addition of 2 N HCl indicated a positive result for the presence of saponins [15].

Flavonoid test. One gram of simplicia powder and one gram of extract were dissolved in 10 mL of distilled water and heated for five minutes using a hot plate. After heating, 0.1 g of magnesium powder and 1 mL of concentrated HCl were added to 5 mL of the filtrate. The mixture was shaken until layers formed. The presence of flavonoids was indicated by a red color change or the formation of an orange-colored layer [15].

Tannin test. One gram of simplicia powder and one gram of extract were heated in 10 mL of distilled water for five minutes, then filtered. Ferric chloride ($FeCl_3$) solution was added to 2 mL of the resulting filtrate. A color change to bluish-black indicated the presence of tannins [15].

Triterpenoid and steroid test. One gram of simplicia powder and one gram of extract were heated for five minutes and then filtered. Two milliliters of the filtrate were mixed with a small amount of ether and shaken. The resulting ether layer was separated, evaporated in a porcelain dish until dry, and then treated with two drops of acetic anhydride and one drop of concentrated sulfuric acid as part of the Liebermann-Burchard reaction. The appearance of a green color indicated the presence of steroids, while red, yellow, or orange coloration suggested the presence of triterpenoids [15].

Test animal treatment

The test animals were 24 healthy male ddY mice (*Mus musculus*), aged 8–12 weeks and weighing 20–40 g, divided into four groups of six animals each. This study received ethical approval from the Health Research Ethics Committee (KEPK) of Jenderal Achmad Yani University, Yogyakarta, with ethical clearance number Skep/663/KEP/XI/2024. The mice were housed in polypropylene cages with rice-husk bedding under standard laboratory conditions (22 ± 3 °C, 50–60% relative humidity, and a 12 h light/dark cycle), with standard pellet feed and drinking water available ad libitum [16]. Prior to testing, the mice underwent a 7-day acclimatization period to adapt to their new environment. This process aimed to minimize stress in the test animals, thereby ensuring more stable and valid responses to the treatments [10].

Prior to the administration of the test preparation, the mice were fasted for 3–4 hours but were allowed free access to drinking water. Subsequently, the body weight of each mouse was measured to determine the appropriate dosage. The animals were divided into four groups: a normal control group receiving 1% CMC-Na and three treatment groups receiving the ethanol extract at doses of 1250, 2500, and 5000 mg/kg BW, respectively. The test preparation was administered as a single oral dose by oral gavage at a volume of 10 mL/kg BW (0.2–0.4 mL per mouse) [16]. Food was reintroduced within 1–2 hours after treatment.

Following administration, each animal was observed individually at consistent times, with at least 30 minutes of close observation immediately after dosing, followed by periodic observations every 4 hours during the first 24 hours. Monitoring continued for 14 days [10]. Observations included food and water intake, changes in body weight, temperature monitoring, diarrhea, diuresis, pharmacological screening, organ index, and mortality. At the end of the observation period, the mice were euthanized by cervical dislocation. After euthanasia, the carcasses were buried to eliminate any potential disease-causing agents and to prevent environmental contamination [10]. Procedures involving animals were performed in accordance with the Indonesian Food and Drug Authority (BPOM) Regulation No. 10 of 2022 concerning guidelines for *in vivo* preclinical toxicity testing [10].

Data analysis

Quantitative data are presented as mean ± standard deviation (SD). Data were analyzed using descriptive and statistical methods. Behavioral changes were evaluated descriptively by comparison with the control group. Quantitative data (body weight, organ index, temperature, and food and water intake) were analyzed statistically using normality (*Shapiro–Wilk*) and homogeneity (*Levene*'s) tests. Parametric analysis (one-way ANOVA followed by *Tukey post hoc* test) was applied for normally distributed and homogeneous data, while non-parametric analysis (Kruskal–Wallis followed by Mann–Whitney test) was used when assumptions were not met. A significance level of $p < 0.05$ was applied [17].

Table 1. Phytochemical screening of cassava tuber peel simplicia and ethanol extract

	Reagents	Results		Description
		Simplicia	Extract	
Alkaloid	Mayer	-	-	No white precipitate was formed with Mayer's reagent
	Dragendorff	-	-	No brown precipitate was formed with Dragendorff's reagent
Saponin	Distilled water + 2 N HCl	+	+	Stable foam was formed
Flavonoid	Mg + HCl	+	+	A color change from brown to orange was observed
Tannin	$FeCl_3$	+	+	A color change from brown to dark blue was observed
Steroids and triterpenoids	Liebermann–Burchard	-	-	No color change to green, orange, or red was observed

(+) Positive: contains the compound group; (–) Negative: does not contain the compound group. $FeCl_3$, iron(III) chloride; HCl, hydrochloric acid; Mg, magnesium powder; N, normality. Qualitative tests were performed on the dried peel powder (simplicia) of cassava (Manihot esculenta Crantz) and on its 70% ethanol extract.

Results

Maceration of 250 g of cassava peel simplicia with 2,500 mL of 70% ethanol yielded 43.79 g of thick extract, corresponding to a yield of 17.52% (w/w).

The results of phytochemical screening of the simplicia and thick extract of cassava tuber peel revealed the presence of flavonoids, saponins, and tannins. In contrast, tests for alkaloids and steroids/triterpenoids showed negative results (Table 1).

Feed consumption did not differ significantly between the control and the three extract-treated groups (Figure 1). The data were normally distributed (Shapiro–Wilk test, $p > 0.05$) and homogeneous (Levene's test, $p > 0.05$), and one-way ANOVA showed no significant differences between groups ($p > 0.05$).

Water consumption was similar in all groups (Figure 2). The data were normally distributed (Shapiro–Wilk test, $p > 0.05$) and homogeneous (Levene's test, $p > 0.05$), and one-way ANOVA showed no significant differences between groups ($p > 0.05$).

Mean body weight increased in all groups during the 14-day observation period, from approximately 28–29 g on day 0 to 30–32.5 g on day 14, with transient day-to-day fluctuations (Figure 3). The data were normally distributed and homogeneous ($p > 0.05$), and one-way ANOVA showed no significant differences between groups ($p > 0.05$).

Rectal temperature ranged from 36.4 to 36.7 °C in all groups at all time points (Table 2). Thirty minutes after dosing, body temperature increased slightly (0.1–0.2 °C) in all groups, including the control. The data were normally distributed (Shapiro–Wilk test, $p > 0.05$) and homogeneous (Levene's test, $p > 0.05$), and differences between groups were not significant at any time point (one-way ANOVA, $p > 0.05$).

No mortality occurred in any group during the 14-day observation period. Grooming was observed in all animals of all groups, including the control, within the first 30 minutes after dosing. No other clinical signs, such as restlessness, aggressiveness, changes in motor activity, tremor, convulsions, changes in respiration, miosis, salivation, decreased grip strength, impaired balance, or limb paralysis, were observed in any group (Table 3). No diarrhea or diuresis was observed during the first 30 minutes after dosing.

No macroscopic abnormalities were observed in any of the organs examined. The relative weights of the lungs, heart, stomach, spleen, liver, kidneys, small intestine, and large intestine in the extract-treated groups were similar to those of the control group (Table 4). The data were normally distributed and homogeneous ($p > 0.05$), and one-way ANOVA showed no significant differences between groups ($p > 0.05$).

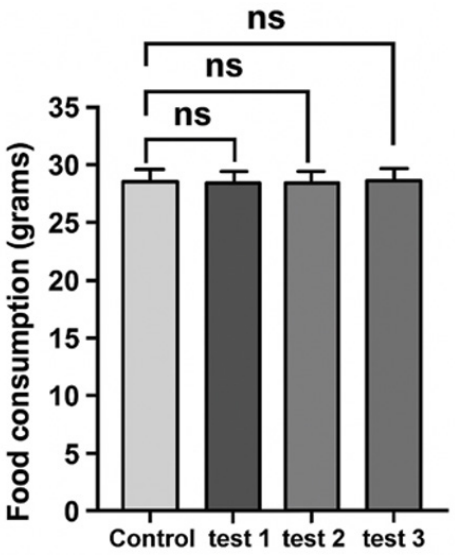


Figure 1. Feed consumption of male ddY mice during the 14-day observation period after a single oral dose of cassava tuber peel ethanol extract. Mice received a single oral dose of 1% sodium carboxymethylcellulose (CMC-Na; control) or the extract at 1250 (test 1), 2500 (test 2), or 5000 mg/kg body weight (BW; test 3). Data are presented as mean ± SD (n = 6). ns, not significant compared with the control (one-way ANOVA, p > 0.05).

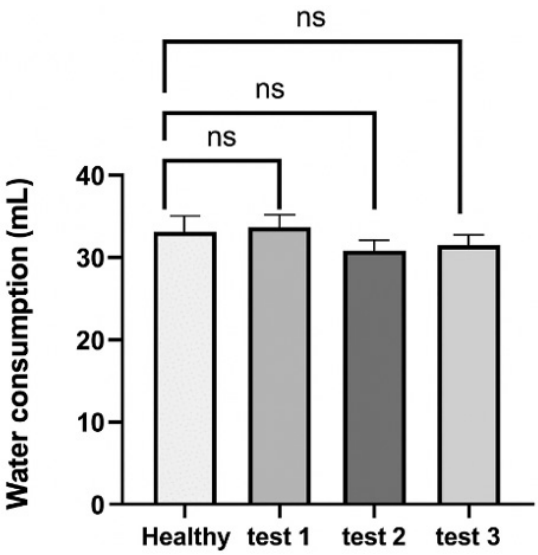


Figure 2. Water consumption of male ddY mice during the 14-day observation period after a single oral dose of cassava tuber peel ethanol extract. Mice received a single oral dose of 1% sodium carboxymethylcellulose (CMC-Na; control) or the extract at 1250 (test 1), 2500 (test 2), or 5000 mg/kg body weight (BW; test 3). Data are presented as mean ± SD (n = 6). ns, not significant compared with the control (one-way ANOVA, p > 0.05).

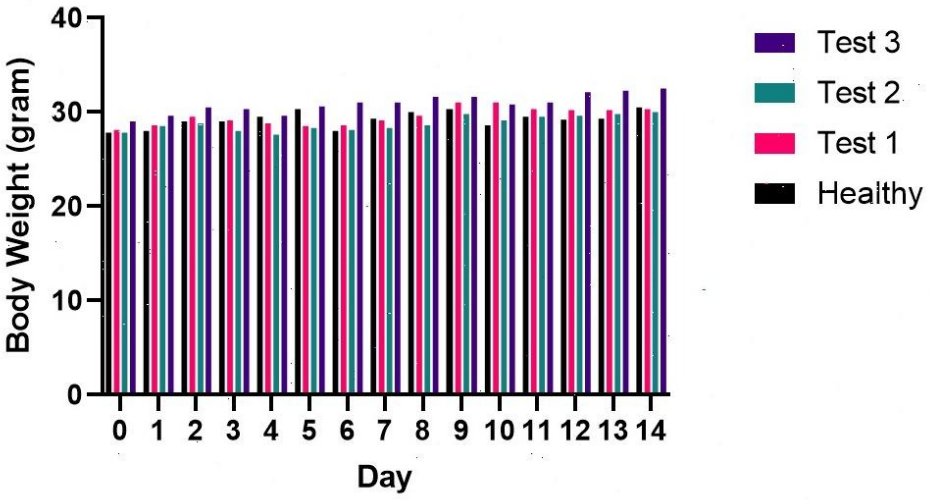


Figure 3. Daily body weight of male ddY mice during the 14-day observation period after a single oral dose of cassava tuber peel ethanol extract. Mice received a single oral dose of 1% sodium carboxymethylcellulose (CMC-Na; control) or the extract at 1250 (test 1), 2500 (test 2), or 5000 mg/kg body weight (BW; test 3). Data are group means (n = 6). Differences between groups were not significant (one-way ANOVA, p > 0.05).

Table 2. Rectal body temperature of male ddY mice before and after a single oral dose of cassava tuber peel ethanol extract

Group	Temperature		
	Acclimatization	Before	30 min after dosing
Control	36.5	36.5	36.6
Test 1	36.5	36.4	36.6
Test 2	36.6	36.6	36.7
Test 3	36.5	36.5	36.6

Control: 1% sodium carboxymethylcellulose (CMC-Na); Test 1: extract 1250 mg/kg BW; Test 2: extract 2500 mg/kg BW; Test 3: extract 5000 mg/kg BW. BW, body weight. Differences between groups were not significant at any time point (one-way ANOVA, $p > 0.05$). Temperature (°C) was measured during acclimatization, immediately before dosing, and 30 min after dosing. Values are group means (n = 6)

Table 3. Clinical signs in male ddY mice after a single oral dose of cassava tuber peel ethanol extract

Types of pharmacological screening	Group			
	Control	Test 1	Test 2	Test 3
Grooming	+	+	+	+
Restlessness	-	-	-	-
Aggressiveness	-	-	-	-
Fear	-	-	-	-
Increased motor activity	-	-	-	-
Decreased motor activity	-	-	-	-
Vasodilation	-	-	-	-
Tremor	-	-	-	-
Clonic and tonic seizures	-	-	-	-
Rapid breathing	-	-	-	-
Tail elevation	-	-	-	-
Miosis	-	-	-	-
Salivation	-	-	-	-
Decreased grip strength	-	-	-	-
Reduced startle response	-	-	-	-
Slow breathing	-	-	-	-
Limb paralysis	-	-	-	-
Vasoconstriction	-	-	-	-
Impaired balance	-	-	-	-
Mortality	-	-	-	-

Control: 1% sodium carboxymethylcellulose (CMC-Na); Test 1: extract 1250 mg/kg BW; Test 2: extract 2500 mg/kg BW; Test 3: extract 5000 mg/kg BW. BW, body weight. Observations were made during the first 30 min after dosing, every 4 h during the first 24 h, and daily thereafter for 14 days; (−): Negative result, indicating no observable effect or absence of the tested parameter in the respective group; (+): Positive result, indicating the presence of an observable effect or the tested parameter in the respective group. Signs were recorded by pharmacological (behavioral) screening of six mice per group during the 14-day observation period.

Table 4. Relative organ weights of male ddY mice 14 days after a single oral dose of cassava tuber peel ethanol extract

Groups	n	Organ							
		Lungs	Heart	Stomach	Spleen	Liver	Kidney	Small intestine	Large intestine
Control	6	0.76±0.11	0.62±0.08	2.02±0.34	0.64±0.10	5.53±0.39	1.80±0.17	6.14±0.81	3.08±0.21
Test 1	6	0.80±0.08	0.61±0.04	2.06±0.63	0.68±0.10	5.53±0.39	1.79±0.10	6.13±1.37	3.10±0.36
Test 2	6	0.72±0.07	0.62±0.13	2.04±0.23	0.62±0.05	5.67±0.22	1.73±0.04	6.19±1.06	3.04±0.50
Test 3	6	0.66±0.08	0.61±0.07	2.07±0.23	0.62±0.08	5.59±0.41	1.76±0.12	6.15±0.35	3.10±0.22

Control: 1% sodium carboxymethylcellulose (CMC-Na); Test 1: extract 1250 mg/kg BW; Test 2: extract 2500 mg/kg BW; Test 3: extract 5000 mg/kg BW. BW, body weight. Organ index (%) = organ weight / body weight × 100. Differences between groups were not significant (one-way ANOVA, $p > 0.05$). The organ index (%) is presented as mean ± SD (n = 6 per group).

Discussion

This study showed that a single oral dose of the ethanol extract of cassava tuber peel at up to 5000 mg/kg BW caused no mortality or clinical signs of toxicity in male ddY mice during the 14-day observation period. Feed and water consumption, body weight, body temperature, and relative organ weights of the treated groups did not differ significantly from those of the control group, and no macroscopic organ abnormalities were found. Therefore, the LD_{50} of the extract is estimated to be greater than 5000 mg/kg BW, and the extract can be classified as practically non-toxic [18].

These findings are consistent with previous safety studies of cassava-derived materials. A crude extract of cassava cortex (peel) containing scopoletin and cyanide had an oral LD_{50} greater than 2000 mg/kg in female Wistar rats, and feeding the cortex powder to broilers did not change biochemical parameters, the relative weights of the liver, kidneys, and brain, or organ histopathology [19]. Similarly, dietary fiber from cassava pulp caused no mortality or clinical signs of toxicity at a single oral dose of 2000 mg/kg in Wistar rats, with an LD_{50} above 2000 mg/kg and a no-observed-adverse-effect level of 1000 mg/kg in a 28-day study [20]. Compared with these studies, which used a maximum dose of 2000 mg/kg, the present study tested a higher dose (5000 mg/kg BW) and extends the evidence of low acute oral toxicity to the 70% ethanol extract of cassava peel, the preparation previously shown to have antidiabetic activity [13].

The extract yield (17.52%) was comparable to those reported for cassava peel extracts in previous studies (16–21%) [6,9]. Flavonoids, saponins, and tannins were detected in both the simplicia and the extract, consistent with previous reports on cassava peel [9,13], and the positive Mg–HCl, $FeCl_3$, and foam tests are characteristic of these compound groups [21]. Flavonoids and tannins are known for their antioxidant properties, which may protect against cellular damage, whereas saponins can cause membrane irritation only at high concentrations. The absence of alkaloids, a group often associated with higher toxicity, is consistent with the low acute toxicity observed. The negative steroid/triterpenoid test despite the presence of saponins may be due to the absence of a hydrolysis step and the limited sensitivity of the test, which can prevent the release and detection of steroid/triterpenoid aglycones from saponin glycosides. A limitation of this study is that alkaloid identification was based only on Mayer's and Dragendorff's reagents, which belong to the same group of precipitation reagents; additional reagents such as Wagner's or Hager's reagents are recommended in future studies.

Cyanogenic glycosides are the main toxicological concern for cassava peel. Their content can be reduced by processing such as sun-drying [22] and heating [23], both of which were applied in this study (sun-drying for three days and concentration at 50 °C). However, linamarin is highly polar and can be readily extracted from cassava peel [24], so residual cyanogenic glycosides may still be present in the 70% ethanol extract. The absence of mortality and of signs of acute cyanide poisoning, such as rapid breathing, convulsions, and impaired balance (Table 3), up to 5000 mg/kg BW suggests that any residual cyanogen content was below the acutely toxic level, in agreement with the low acute toxicity of a cyanide-containing cassava cortex extract [19]. Because the cyanogenic glycoside

content of the extract was not measured in this study, its determination is recommended in future studies, particularly before repeated-dose toxicity testing.

Feed and water consumption remained within the normal ranges reported for mice (3–5 g and 6–7 mL per mouse per day, respectively) [25] and did not differ between groups, indicating that the extract did not affect appetite, energy homeostasis, or fluid balance [26]. Body weight is a sensitive indicator of general toxicity; the transient decreases observed did not exceed 10% of the initial body weight and were not accompanied by abnormal behavior, which is within the limit considered non-toxic [27]. Such fluctuations can be caused by handling stress and environmental conditions [28,29].

The slight increase in body temperature 30 minutes after dosing occurred in all groups, including the control, and was therefore most likely related to handling and gavage stress rather than to the extract. It did not exceed 1 °C above the normal body temperature of mice and therefore cannot be considered fever [30,31]. Diarrhea and diuresis, which may indicate parasympathetic stimulation [31], were not observed. The transient grooming seen in all groups, including the control, is attributed to contact of the dosing preparation with the fur around the mouth [18,32] and is regarded as a non-adverse response to dosing [16].

Relative organ weight is a sensitive indicator of organ hypertrophy or atrophy after toxic exposure [10]. The liver, kidneys, heart, lungs, spleen, and gastrointestinal tract are the main targets of systemic toxicity and of local irritation after oral administration [16,33,34]. The absence of significant changes in their relative weights, together with their normal macroscopic appearance, indicates that the extract did not cause organ damage at the doses tested. However, histopathological, hematological, and biochemical examinations were not performed in this study and should be included in subsequent subchronic toxicity studies.

Conclusion

A single oral dose of the ethanol extract of cassava tuber peel up to 5000 mg/kg BW produced no mortality, no clinical signs of toxicity, and no significant changes in body weight, body temperature, feed and water intake, or organ indices in male ddY mice. The LD_{50} of the extract is therefore estimated to be greater than 5000 mg/kg BW, and the extract can be classified as practically non-toxic. These findings support the further development of cassava peel extract as a herbal medicinal ingredient; however, subchronic toxicity and histopathological studies are needed to confirm its safety for long-term use, such as in antidiabetic therapy.

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Conflict of interest

The authors declare that there is no conflict of interest in the conduct of this research and the preparation of this manuscript.

Author contributions

ASMBN: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. DDAK: Conceptualization, Methodology, Supervision, Project administration, Resources, Writing – review & editing. RH: Methodology, Validation, Supervision, Writing – review & editing. All authors read and approved the final manuscript.

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Declaration of generative AI use

The authors used DeepL to translate the manuscript and refine its grammar. The translated text and grammatical revisions were then manually checked by the authors.

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