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Effect of fermented cassava (*Manihot esculenta* Crantz) on maternal development and fetal teratogenicity in mice (*Mus musculus* L.)

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ABSTRACT

Fermented cassava (*Manihot esculenta* Crantz) is one of the traditional foods favored by the community, including pregnant women, but it has been reported to have various adverse effects. Therefore, this study aimed to analyze the effect of fermented cassava on maternal development and fetal teratogenicity in mice *(Mus musculus* L.). A total of 36 pregnant female white mice were divided into three main groups representing different pregnancy periods (first, second, and third). Each main group was subdivided into four subgroups, including a control group and three treated groups. The treated groups received doses of 0.075 g/kg, 0.15 g/kg, and 0.3 g/kg body weight of fermented *M. esculenta* Crantz. Furthermore, the treatment was orally administered for five consecutive days, with daily measurements of maternal body weight. On the 18th day of pregnancy, mice were laparatomized to evaluate the morphological and skeletal teratogenic effects of fermented cassava on the fetus. Statistical analysis of maternal body weight, number of fetuses, and fetal body weight during pregnancy was conducted. The administration of fermented cassava significantly affected maternal body weight. The results showed that there was no significant difference between the number of fetuses and fetal body weight. However, the intake of fermented cassava in a dose-dependent manner tended to reduce the number of fetuses and fetal weight, compared to the control group in all gestation periods. Based on the results, fermented cassava exhibited a potential teratogenic effect on certain fetuses, leading to spontaneous abortion and fetal death in all treated groups and gestation periods, particularly in the second period. Therefore, its consumption is deemed unsafe and must be prohibited for pregnant women.

Keywords: fermented cassava; *Manihot esculenta* Crantz; maternal development; fetal teratogenicity; *Mus musculus* L.

1. INTRODUCTION

Cassava or tapioca (*Manihot esculenta* Crantz) is a woody shrub belonging to the Euphorbiaceae family. Furthermore, it is one of the major staple food crops in several developing countries and serves as a primary source of starch. Cassava is primarily cultivated in tropical and subtropical regions across continents, such as Africa, Asia, and Latin America (Bahekar and Kale, 2015; Manthey, 2016). As an invaluable source of carbohydrates and energy, it holds a significant presence in the Indonesian agriculture landscape. According to previous studies, the methods of consumption for fermented cassava vary, allowing a range of culinary applications such as being consumed fresh or immediately after boiling or frying. In other areas, peeled cassava starch is a key ingredient in the production of a solid fermented cassava product, known as tape singkong (Frediansyah, 2017; Cempaka, 2021).

Tape singkong represents a traditional Indonesian culinary product that is widely known for its distinctive sweet-sour taste and alcoholic properties. The preparation process comprises peeling, steaming, or boiling cassava for approximately 2.5 h. Subsequently, cassava is cut into pieces and sprinkled with powdered yeast before being placed in woven bamboo baskets lined with banana leaves. These baskets are then kept in a shaded location for 1-3 days until a dense white mycelium is formed on the surface (Nicolau, 2016). Cassava roots and leaves have been reported to contain cyanogenic glucosides, including linamarin and hydrocyanic acid, at levels that can be toxic when consumed in the fresh state. However, appropriate processing techniques, such as roasting, soaking, and fermentation, can reduce cyanide concentration to negligible levels (Blagbrough et al., 2010; Nicolau, 2016).

Fermented cassava is typically derived from the fermentation process, in which starch is mixed with the yeast *Saccharomyces cerevisiae* (Hidayat et al., 2018). During the fermentation, *S. cerevisiae* engages in a fermentative metabolism facilitated by sugars and other essential nutrients, including amino acids, minerals, and vitamins. This metabolic activity often results in the production of ethanol and carbon dioxide as the primary metabolites (Walker and Stewart, 2016). The nutritional value of cassava commonly increases after fermentation, particularly in terms of the protein content. Furthermore, protein is essential for supporting the body's growth and development, repairing damaged tissue cells, and producing digestive and metabolic enzymes (Nicolau, 2016). Every 100 g of fermented cassava contains 160 kcal of energy, 0.3 g of fat, 1.4 g of protein, 1.8 g of fiber, 16 mg of calcium, 27 mg of phosphorus, 271 mg of potassium, 20 mg of ascorbic acid, and 11 µg-DFE of folate (Chandrasekara and Kumar, 2016). In addition, this product has also been reported to be a source of lactic acid bacteria (LAB), which not only increases the nutritional quality but also functions as an antimicrobial and probiotic in the digestive tract (Aly et al., 2016).

In Indonesia, pregnant women are prohibited from consuming fermented cassava due to the belief that it may cause miscarriage (Wibowo et al., 2016; Suriah and Adriani, 2022). Although the alcohol content in fermented cassava is generally low, it becomes a matter of concern when consumed excessively during pregnancy. Several studies showed that the alcohol content is influenced by the quantity of yeast added and the duration of fermentation. Hasanah et al., (2012) stated that prolonged fermentation of cassava correlated with higher ethanol content. Duration of 24 to 120 h also led to ethanol contents ranging between 0.84 to 11.81%.

Pregnancy is a vulnerable period, where environmental factors can have a significant impact on both the mother's health and the safety of the developing fetus, particularly at the organogenesis stage (Kenner, 2019). One such harmful influence is alcohol consumption during pregnancy, which has been shown to increase the risk of adverse birth outcomes, particularly preterm delivery and low birth weight (Addila et al., 2021). Sundermann et al. (2021) also reported that drinking alcohol increased the risk of spontaneous abortion (miscarriage).

According to previous studies, the administration of teratogenic chemicals during the preconception, prenatal, or postnatal stage can lead to developmental toxicity. This term comprises a range of adverse outcomes, including mortality of the developing organisms, anatomical anomalies, disrupted growth patterns, and functional deficiencies (Duong et al., 2011). Therefore, this study aimed to determine the adverse effects of fermented cassava on maternal development and fetal teratogenicity.

2. MATERIALS AND METHODS

2.1 Plant collection and identification

Fermented cassava (*M. esculenta* Crantz) was purchased from traditional market in Padang, West Sumatra, Indonesia. The plant material was then identified in the Herbarium of Universitas Andalas, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, with voucher specimen number 289/K-ID/ANDA/VIII /2020.

2.2 Preparation

The fermented cassava extract was prepared in doses of 0.075 g/kg, 0.15 g/kg, and 0.3 g/kg for the treatment groups by dissolving it in distilled water, while aquades was used for the control group. Furthermore, the test preparation was given orally at a dose of 0.4 mL/20 g. Bouin solution was then prepared by mixing 25 mL of formaldehyde with 5 mL of glacial acetic acid and 75 mL of saturated picric acid (Hayes and Kruger, 2014). Alizarin red solution was prepared by adding 6 mg of red alizarin in one liter of 1% aqueous KOH (Manson et al., 1982; Rusdi et al., 2021).

2.3 Experimental animal

The sample population consisted of female mice (*Mus Musculus* L.), aged 2–3 months, obtained from the animal center, Universitas Andalas, with an average weight of 20–30 g. The animals were housed in an environmentally controlled room with access to food and water ad libitum. The procedures were performed strictly to institutional protocols and followed the animal care provisions outlined by the ethics committee of the Faculty of Medicine, Universitas Andalas (No. 112/UN.16.2/KEP-FK/2020).

2.4 Mating procedure

Virgin healthy female mice in the proestrus phase were paired overnight with males at a ratio of 4:1. The presence of a vaginal plug the next day showed successful mating and was designated as day 0 of pregnancy.

2.5 Treatment of the animals

A total of 36 pregnant mice were divided into three main groups corresponding to the first, second, and third periods. Each main group was further subdivided into four subgroups, consisting of a control and three treated groups. The treated groups received doses of fermented *M. esculenta* Crantz at various concentrations of 0.075 mg/kg, 0.15 mg/kg, and 0.3 mg/kg. Each experimental group comprised three pregnant mice, and the dosage levels were established based on the daily maximum carbohydrate intake in humans as outlined by Burke et al. (2001), which were then adjusted for the samples. Following the protocol established by Nazar et al. (2019), the treatment was administered orally for five consecutive days during each treatment period (1–5 days for the first period, 7–11 days for the second period, and 13–17 days for the third period).

2.6 Maternal observation

During the gestational period, mice were monitored at least once daily for any behavioral changes, signs of pharmacological effects, or general changes in health. Mortality, morbidity, abortion, or premature delivery events were documented, along with daily recordings of the body weight. Furthermore, vaginal bleeding during pregnancy was identified to detect any signs of spontaneous abortion or miscarriage. Pregnancy index and percentage of spontaneous abortion were obtained using Equations 1 and 2 (Weidner and Sigwart, 2000).

$$Pregnancy\ index = \frac{the\ number\ of\ pregnant\ parent\ mice}{number\ of\ vaginal\ plug\ positive\ females\ (mated\ females)}\ x\ 100 \quad (1)$$

$$Pencentage\ of\ spontaneous\ abortion\ = \frac{total\ aborted\ pregnant\ mice}{total\ pregnant\ pregnant\ mice}\ x\ 100 \quad (2)$$

2.7 Teratogenic effect and fetal development parameters

Teratogenic studies were performed in the second period using the methods described by Marsden and Leroy (2013), with slight modifications. On the 18th day of pregnancy, the samples were sacrificed by neck dislocation, and laparotomy was performed. The uterine and ovaries were dissected, where the live and dead fetuses and resorptions were counted. The fetuses were carefully removed from the uterus and assessed macroscopically for external malformations and visual abnormalities, such as the tail, earlobe, eyelid, number of fronts, and rear fingers. The body weight of the animals was also measured during the process. Alternate fetuses in the uterine horns were apportioned for either skeletal or visceral examinations. Samples assigned to skeletal examination were dissected and stained with Alizarin Red solution for 2–3 days. The fetuses subjected to visceral examination were immersed in Bouin fixative for 14 days and were dissected on a free-hand basis. The percentage of live and dead samples was calculated using Equations 3 and 4 (Weidner and Sigwart, 2000).

2.8 Statistical analysis

The results of the statistical analysis were presented as mean values±SD. A two-way analysis of variance (ANOVA) was used to determine the statistical significance between group treatments and gestational age, followed by Duncan multiple range test. The difference was considered statistically significant at $p<0.05$.

$$Pencentage\ of\ live\ fetuses\ = \frac{total\ live\ fetuses}{total\ fetuse}\ x\ 100 \quad (3)$$

$$Pencentage\ of\ dead\ fetuses\ = 100 -\ Pencentage\ of\ live\ fetuses \quad (4)$$

3. RESULTS

3.1 Prenatal evaluation of maternal parameters

In the first period, the body weight of pregnant mice was significantly ($p<0.05$) affected by the treatment groups and the gestational age. In the second and third periods, the body weight was not significantly different within the treatment groups, but statistically different ($p<0.05$) in terms of gestational age, compared to controls. The interaction between these factors did not considerably affect the mean pregnant mice body weight ($p>0.05$) in all gestation periods (Figure 1 and Table 1).

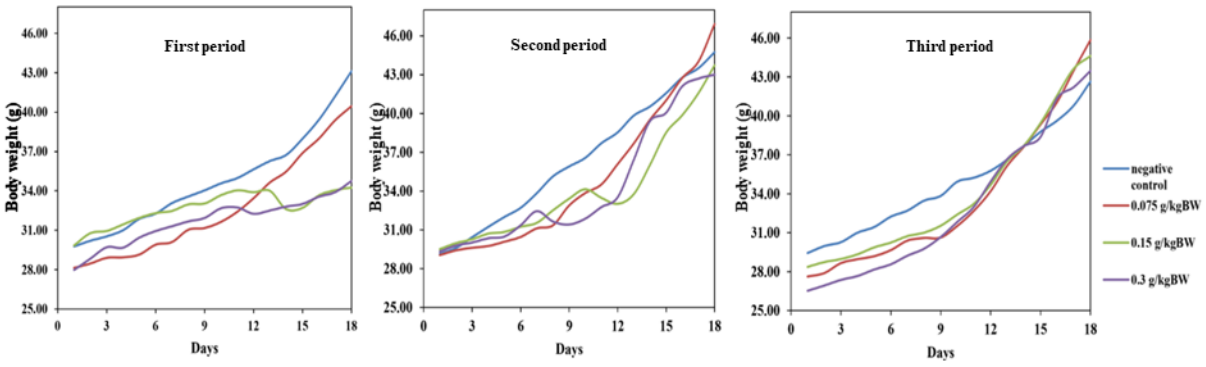

Figure 1. Impact of fermented cassava on the parent mice's body weight during pregnancy

Table 1. The impact of fermented cassava on parent mice body weight during pregnancy

Period	First					Second					Third				
Days	**D0**	**D1**	**D2**	**D3**	**Mean±SD**	**D0**	**D1**	**D2**	**D3**	**Mean±SD**	**D0**	**D1**	**D2**	**D3**	**Mean±SD**
2	30.20±0.50	28.47±1.63	30.77±2.59	28.83±2.36	29.57±1.09^{p}	29.60±3.37	29.40±3.10	29.97±2.55	29.77±3.37	29.68±0.24^{p}	29.97±1.77	27.90±2.51	28.73±2.00	26.93±2.77	28.38±1.29^{p}
4	31.00±0.44	28.93±1.16	31.43±2.72	29.67±1.89	30.26±1.16^{pq}	31.23±3.63	29.73±3.40	30.70±2.79	30.33±3.21	30.50±0.63^{p}	31.00±1.68	28.97±2.50	29.33±1.89	27.67±2.60	29.24±1.37^{pq}
6	32.27±1.36	29.90±1.56	32.30±3.67	30.93±2.81	31.35±1.16^{pqr}	32.70±3.82	30.43±3.71	31.23±3.10	31.33±3.73	31.43±0.94^{pq}	32.23±2.15	29.67±2.27	30.23±2.54	28.57±2.29	30.18±1.54^{pq}
8	33.57±1.53	31.03±1.08	32.97±4.17	31.67±3.59	32.31±1.16^{pqrs}	35.13±3.69	31.33±2.58	32.50±4.59	31.63±3.45	32.65±1.73^{pq}	33.47±2.08	30.60±2.15	31.00±3.08	29.77±1.76	31.21±1.59^{qr}
10	34.57±1.40	31.63±0.95	33.67±4.20	32.67±4.48	33.13±1.27^{qrs}	36.57±3.33	33.83±3.19	34.13±5.56	31.85±1.48	34.10±1.93^{qr}	34.93±2.06	31.53±2.25	32.40±2.85	31.83±0.97	32.68±1.55^{r}
12	35.63±1.90	33.43±1.08	33.90±4.30	32.25±7.00	33.80±1.40^{rst}	38.53±3.81	36.07±3.01	33.00±1.84	33.50±1.70	35.28±2.55^{r}	35.77±2.08	34.20±2.57	34.67±2.93	34.93±1.11	34.89±0.66^{s}
14	36.73±1.96	35.47±2.45	32.60±4.53	32.80±7.35	34.40±2.03st	40.53±3.07	39.50±2.67	36.00±4.95	39.40±2.55	38.86±1.97^{s}	37.67±2.93	37.63±2.87	37.70±1.97	37.67±1.01	37.67±0.03^{t}
16	39.40±1.90	37.97±3.86	33.65±5.73	33.55±8.27	36.14±2.99^{tu}	42.77±2.00	42.73±2.08	39.85±3.04	42.10±2.40	41.86±1.38^{t}	39.63±2.40	41.07±2.57	41.57±2.27	41.35±0.49	40.90±0.87^{u}
18	43.10±1.85	40.43±4.98	34.25±7.14	34.75±10.11	38.13±4.34^{u}	44.73±1.77	46.90±3.12	43.70±4.38	43.00±1.41	44.58±1.70^{t}	42.63±2.67	45.83±3.65	44.60±1.87	43.45±1.20	44.13±1.39^{v}
Mean±SD	34.79±1.40^{b}	32.67±1.83^{a}	32.70±3.50^{a}	31.70±3.94^{a}		39.22±3.09^{b}	37.63±2.67^{ab}	33.80±2.11^{a}	36.25±1.19^{a}		34.80±2.12^{b}	33.65±2.58^{b}	34.03±1.98^{b}	33.02±2.70^{a}	

Note: Values are presented as mean ± SD.
Significant values were obtained statistically from two-way ANOVA and Duncan' s multiple range T-test.
Different superscripts denote statistical significance ($p < 0.05$).
D0 = negative control, D1 = 0.075 g/kg BW, D2 = 0.15 g/kg BW, D3 = 0.3 g/kg BW of fermented cassava.

The mean body weight in the pregnant mice continued to increase along with the gestational age, while the weight of the control was reported to be higher compared to the treated groups. However, there were no differences between the treated groups. Vaginal bleeding and spontaneous abortion were reported at 0.15 and 0.3 mg/kg BW doses in all gestation periods (Figure 2). Furthermore, the ratio of pregnant per vaginal plug-positive females (pregnancy index) was 67.67% in the first period, as shown in Table 2.

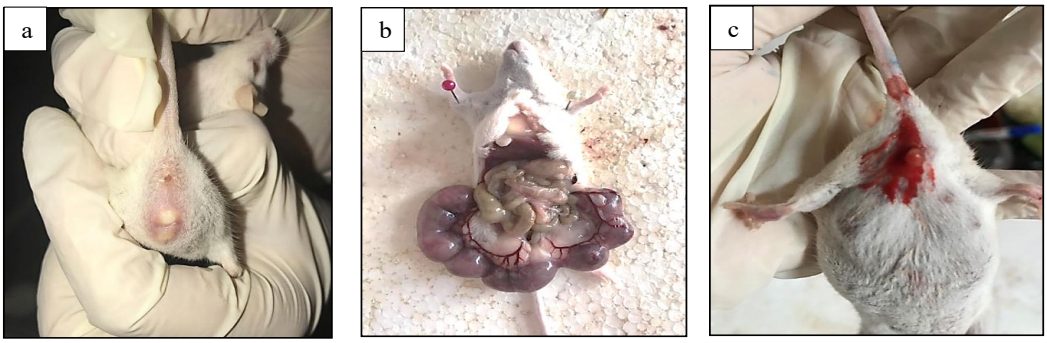

Figure 2. a) Vaginal plug-positive females (mated females), b) observation after laparotomy, and c) vaginal bleeding indicating spontaneous abortion

Table 2. Maternal parameter index

Maternal parameter	First period				Second period				Third period			
	D0	D1	D2	D3	D0	D1	D2	D3	D0	D1	D2	D3
Mated females (n)	3	3	3	3	3	3	3	3	3	3	3	3
Pregnant mice (n)	3	3	2	2	3	3	3	3	3	3	3	3
Pregnancy index (%)	100	100	66.67	66.67	100	100	100	100	100	100	100	100
Spontaneous abortion (%)	0	0	100	33.33	0	0	33.33	33.33	0	0	0	33.33

Note: D0 = negative control, D1 = 0.075 g/kgBW, D2 = 0.15 g/kgBW, D3 = 0.3 g/kgBW of fermented cassava.

3.2 Fetal parameter

The two-way analysis of variance (ANOVA) showed that the number of fetuses and fetal body weight did not statistically affect the treatment groups, gestation periods, and the interaction between these variables (p>0.05) (Figures 3 and 4).

The number of fetuses and fetal body weight played a role in maternal body weight. Although the maternal body weight showed statistical differences in all gestation periods, compared to the control group, the fetal body weight and the number of fetuses were lower at doses of 0.15 mg/kg BW and 0.3 mg/kg BW in the first and second periods (Figure 4).

The results showed that the number of dead fetuses increased along with higher dosage treatment in all gestation periods. Dead fetuses were found at 0.15 g/kg BW and 0.3 g/kg BW doses in the first and second periods and at a dose of 0.3 g/kg BW in the third period, as shown in Figures 5 and 6.

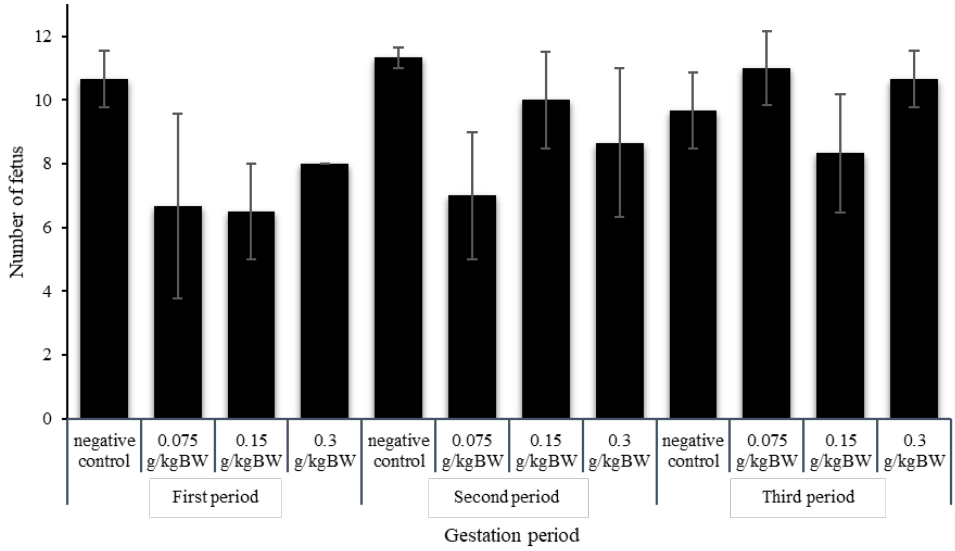

Figure 3. Impact of fermented cassava on the number of fetuses

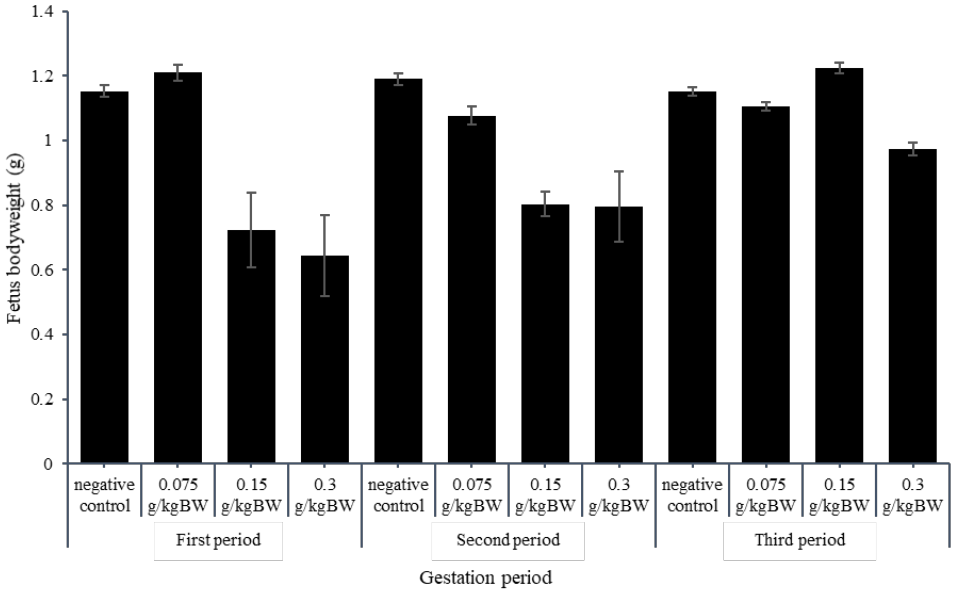

Figure 4. Impact of fermented cassava on the fetal body weight

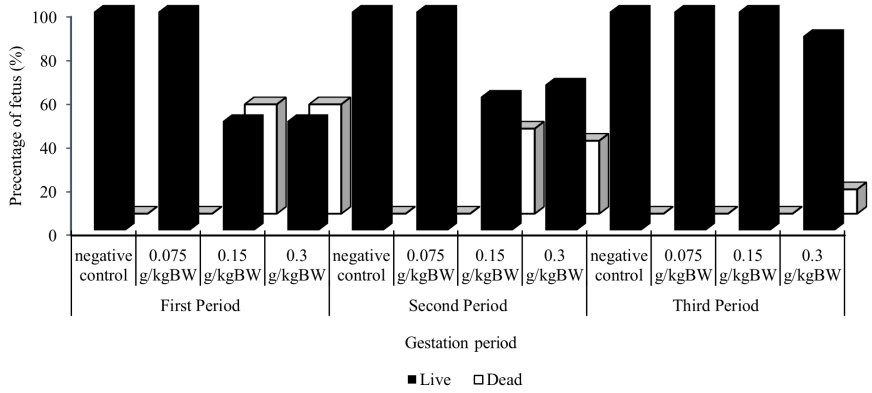

Figure 5. Impact of fermented cassava on the number of live/dead fetuses

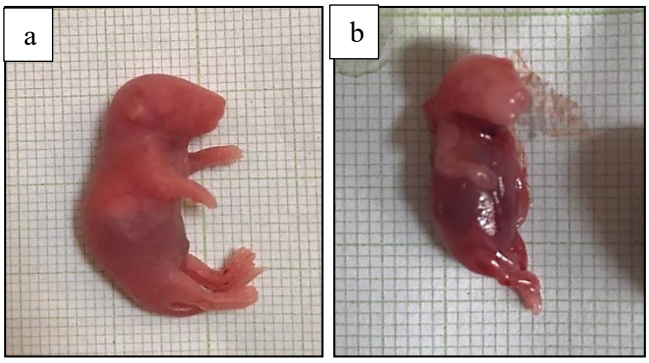

Figure 6. a) Live fetus and b) dead fetus

3.3 Teratogenicity

This study found no defects in mice fetuses in all treated and control groups. The difference in the dose of fermented cassava given to pregnant mice did not cause physical defects or abnormalities in the fetus. Furthermore, the eyelids, ears, tail, feet, toes, and cleft palate were found to be normal. A similar result was also obtained for the number and shape of bones in all treatment groups (Figure 7).

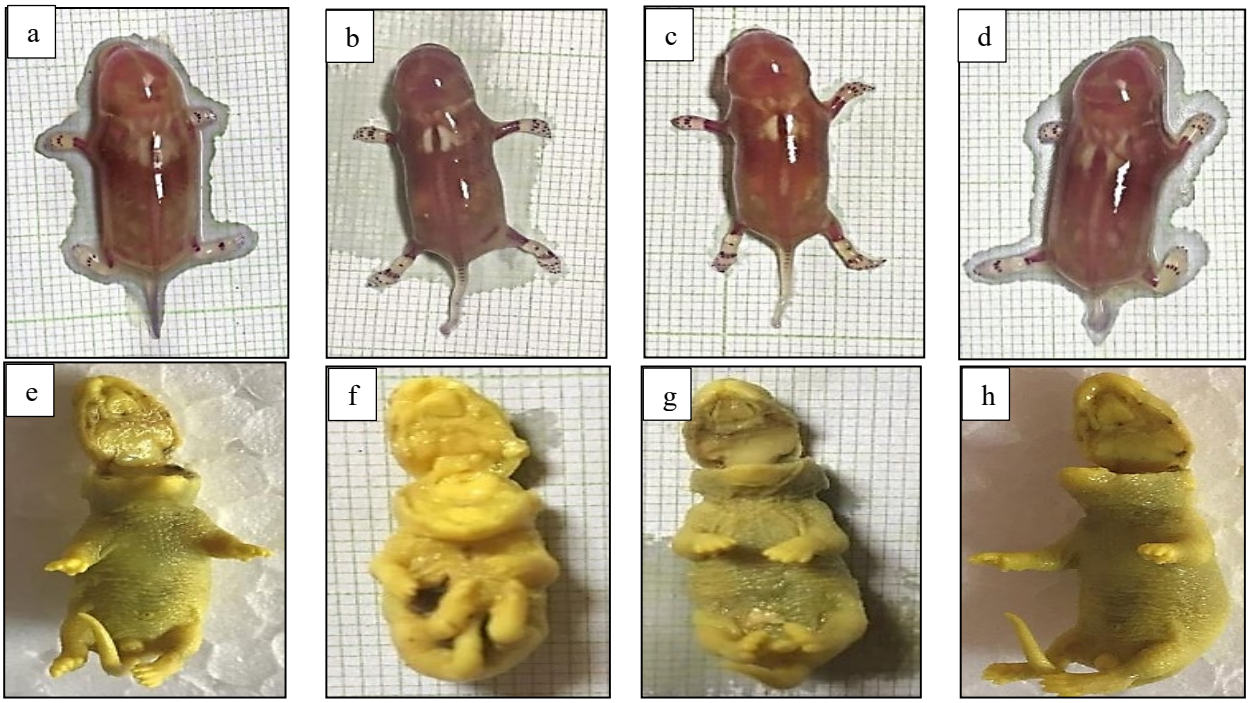

Figure 7. Skeletal and visceral observation; a) negative control, b) 0.075 g/kg BW, c) 0.15 g/kg BW, d) 0.3 g/kg BW of fermented cassava, and visceral examination; e) negative control, f) 0.075 g/kg BW, g) 0.15 g/kg BW, h) 0.3 g/kg BW of fermented cassava

4. DISCUSSION

Over the years, there has been a belief in Indonesia against the consumption of fermented cassava by pregnant women. Fermented cassava is a processed food with carbohydrate starch mixed with yeast *Saccharomyces cerevisiae* to produce alcohol (ethanol) and carbon dioxide (Hidayat et al., 2018). The variant used in this study was obtained commercially from a traditional market and subjected to a 3 to 4-day fermentation process, resulting in unique attributes such as a distinctive texture and a sweet-sour taste, making it suitable for consumption. This was consistent with a previous study, where the length of fermentation had a significant impact on the ethanol content of the product (Hasanah et al., 2012). A duration between 24 and 120 h produced ethanol contents with a range of 0.84 to 11.81%.

Fresh cassava contains various compounds, including cyanogenic glucosides (e.g., linamarin and lotaustralin), non-cyanogenic glucosides, hydroxycoumarins (such as scopoletin), steroidal compounds, terpenoids, and flavonoids (Blagbrough et al., 2010; Emmanuel et al., 2012). Although the compounds possess medicinal properties, it is crucial to note that their content also produces antinutritional factors, such as cyanide, oxalate, phytate, tannin, and trypsin inhibitors. The presence of these factors could interfere with nutrient absorption in the body (Blagbrough et al., 2010; Jamil and Bujang, 2016). Among the antinutrients, cyanide stands out as the most toxic compound, significantly limiting the consumption of cassava tubers and leaves. However, proficient processing techniques, such as fermentation, have shown their effectiveness in reducing the compound's concentration in these tubers to negligible levels (Blagbrough et al., 2010; Chandrasekara and Kumar, 2016; Nicolau, 2016). This finding is consistent with a previous study that compared cyanide levels in raw and fermented cassava, showing the absence of the compound traces through argentometric analysis after fermentation (Budiman et al., 2021).

The study procedures were carried out in mice models due to their small size, short gestation, large litter size, ease of breeding, and availability (Rousseaux and Bolon, 2013). Alcohol consumption in pregnancy poses specific risks to the developing fetus. Furthermore, it has also been linked to several adverse health effects, including stillbirth, miscarriage, preterm labor, and intrauterine growth restriction, which could contribute to additional morbidity associated with any underlying disability, such as low birth weight (Allebeck and Olsen, 1998; Dejong et al., 2019), as found in this study. After consumption, fermented cassava produces ethanol that can readily pass through the placenta and rapidly disperse into the fetal compartment, accumulating in the amniotic fluid. This accumulation damaged the developing fetus and embryo, leading to spontaneous abortion (Sundermann et al., 2019; Tai et al., 2017). The results showed that parent body weight increased along with gestational age, fetal number, and fetal body weight. However, the value obtained in the treated groups was lower, compared to the control. The fetal number and body weight in all periods were also found to decrease along with increasing dose, compared to the control, but it was not significantly different.

Fetal development of mammals comprises a sequence of phases, including blastocyst formation, gastrulation, organogenesis, histogenesis, and function maturation (Jamkhande et al., 2014). In mice, blastocyst formation and gastrulation spanned until the 6th day, and organogenesis was completed on the 15th day, while the histogenesis and maturation continued in the postnatal period. The teratogenic susceptibility of the embryo was still relatively

low during the gastrulation phase. Furthermore, exposure to toxicants did not lead to structural malformations in this developmental stage. A previous study reported that embryonic death, resorption, and frequency of malformations were higher in spontaneous abortions, compared to liveborn infants, reflecting the incompatibility of more severe issues with survival (Jamkhande et al., 2014; Rousseaux and Bolon, 2013).

The results were in line with the present study, where higher doses of fermented cassava were reported to cause spontaneous abortion and fetal death in blastocyst formation and gastrulation (first period). Organogenesis, dominantly occurring in the second period, was an essential phase. Exposure to exogenous toxicants could produce catastrophic effects on the growing organism, leading to significant abnormalities (Rousseaux and Bolon, 2013). Based on the results, a dose of 0.15 g/kg BW was able to cause spontaneous abortion and fetal death. As organogenesis neared completion, the risk of malformation decreased, tending to require higher dose, which led to increased susceptibility to functional impairment. Furthermore, a dose of 0.3g/kg BW was found to cause spontaneous abortion and fetal death in the latter period.

This study found no physical defects or abnormalities in mice fetuses in the treated groups, compared to the control. However, reports on other animal models have indicated that various structural abnormalities, including those affecting the brain, could arise from alcohol exposure confined to a specific period during embryonic development (Lipinski et al., 2012). Excessive cell death, changes in cell cycle, proliferation, migration and morphogenesis, altered gene expression, and disruption of cell signaling were among the identified contributors to the cascades of alcohol-induced developmental impairment (Goodlett et al., 2005). Multiple pathways have also been proposed as mechanisms for alcohol-induced oxidative stress, with NADPH oxidase (NOX) as a potential source of radical oxygen species (ROS). Alcohol exposure has been shown to stimulate NOX enzyme activity and enhance the mRNA expression of NOX regulatory subunits (Sulik, 2014).

Establishing a dose-response relationship between prenatal alcohol consumption and fetal/neonatal outcomes is a complex challenge due to confounding variables, such as maternal alcohol clearance rates, fetal developmental sensitivity, genetic susceptibility, and the timing and duration of consumption during pregnancy (Dejong et al., 2019). Consequently, alcohol was considered to be teratogenic throughout all three trimesters, and intake at any point during pregnancy had the potential for irreversible harm to fetuses. This study failed to provide evidence of a safe level of fermented cassava during pregnancy, and there was no safe timeframe for the consumption of alcohol during gestation without significant risk. Therefore, it is recommended that pregnant women and women attempting to conceive must completely avoid consumption of fermented cassava (Vorgias et al., 2023).

5. CONCLUSION

In conclusion, the administration of fermented cassava in all treated groups and gestation periods could affect the development of pregnancy and reduce the number of fetuses and fetal weight, but there was no statistical difference. The fermented cassava had a potential teratogenic effect on some fetuses, including spontaneous abortion and fetal death in all treated groups and gestation periods, specifically in the second period. Therefore, it could be concluded that the consumption of fermented cassava is unsafe and should be prohibited for pregnant women.

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