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Analysis of the Combination of Sago Grub Flour (*Rhynchophorus ferrugineus*) and Moringa Leaf Flour (*Moringa oliefera L*) in Increasing Hemoglobin Levels in Mice

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Abstract

Anemia is a global health problem that contributes to increased morbidity and mortality, decreased work productivity, and impaired neurological development. The most common type of anemia is iron deficiency anemia. Traditional/local foods can be utilized to address the problem of anemia. Food ingredients that can be used as natural ingredients to overcome anemia include sago grubs and moringa leaves. Sago grubs (*Rhynchophorus ferrugineus*) have a high content of protein, amino acids, iron, and good fats. Moringa leaves (*Moringa oliefera L*) are known to have nutritional content of iron, protein, vitamin A, vitamin C, potassium, and calcium, which are very important in increasing hemoglobin. This study aims to analyze the effect of giving a combination of sago grub flour and moringa leaf flour on hemoglobin levels in anemic mice. This study used sago grubs as the main ingredient for making sago grub flour, moringa leaf flour, and 30 female BALB/c mice divided into 5 groups: PC, NC, T1 (25% sago grub flour: 75% moringa leaf flour), T2 (50% sago grub flour: 50% moringa leaf flour), and T3 (75% sago grub flour: 25% moringa leaf flour). Statistical analysis was performed using the one-way ANOVA test and the results obtained were p=0.000 (p<0.05), then the Post Hoc test was performed and the results obtained were T1 (p=0.000), T2 (p=0.004), and T3 (p=0.125), which means there is an effect of the combination of sago grub flour and moringa leaf flour on increasing Hb levels in anemic mice. However, of the three treatments, the most influential group was the T3 group, which was able to increase hemoglobin from 6.3 mg/dl to 15.36 mg/dl.

Keywords: Anemia, hemoglobin levels, moringa leaves, sago grubs

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1. Introduction

Anemia is a global health issue [1], affecting all age groups, from infants and children to adolescents and the elderly [2], [3]. Anemia is a condition where hemoglobin (Hb) levels in the blood are lower than normal [4]. It is also defined as a decrease in the concentration of red blood cell mass or blood hemoglobin and hematocrit [5]. One of the causes of anemia is iron deficiency, where iron is a macro mineral needed by the body and plays a role in the formation of hemoglobin[6]. Globally, anemia affects about 40% of children [1] and 30% of women of reproductive age [7]. One in two pregnant women is reported to suffer from anemia. More than two billion people, or over 30% of the world's population, suffer from anemia caused by iron deficiency [3]. Iron deficiency occurs due to an imbalance between intake and the body's needs [8]. Anemia caused by iron deficiency occurs almost worldwide, especially in developing countries [9], including Indonesia. Indonesia is one of the countries in Southeast Asia with a high prevalence of anemia. In 2023, the prevalence of anemia in pregnant women was 27.7%, in toddlers 23.8%, and in children 16.3% [10]. Anemia affects one-third of the world's population and contributes to increased morbidity and mortality, decreased work productivity, and impaired neurological development [11]. Anemia is a condition where there is a decrease in the number of red blood cells in the body, characterized by hemoglobin levels in men less than 13.0 g/dL and in women less than 12.0 g/dL [12]. A common symptom in people with anemia is paleness, most noticeable on the palms, nail beds, and conjunctiva [6]. Other clinical signs of anemia include palpitations, shortness of breath during activity, headaches, tinnitus, vertigo, and syncope. Adolescents may experience fatigue, coldness, decreased cognitive function, and dizziness. If hemoglobin levels are too low, it can cause loss of appetite, restlessness, lethargy, tachycardia, and heart failure [13]. Anemia in children impacts their psychomotor development and cognitive abilities [14], as well as their susceptibility to infections [15]. In women of childbearing age, it can lead to decreased immunity, making them prone to infections, reduced fitness and mental agility due to lack of oxygen to muscle and brain cells, and decreased academic achievement and work productivity. These impacts can carry over to pregnancy, where anemia increases the risk of stunted fetal growth, premature birth, low birth weight, impaired child development, bleeding before and during childbirth (threatening the safety of both mother and baby), and low iron stores in newborns, leading to anemia in infancy and early childhood. It also increases the risk of neonatal and infant morbidity and mortality [2] .

To recover from anemia, the body needs adequate intake of iron and protein. Protein plays an important role in the formation of blood cells like red blood cells and hemoglobin, and it functions in transporting iron for blood cell formation in the bone marrow[16]. As transferrin, protein transports iron from the digestive tract through the intestinal wall into the blood via cell membranes[17]. One traditional food that can be used as a substitute for animal protein is the sago grub. Sago grubs (Rhynchophorus ferrugineus) belong to the order Coleoptera and are categorized as safe for consumption based on toxicological evaluations of edible insects[18]. Research has shown that edible insects are identified as viable sources of traditional protein for consumption, thus supporting future food security and sustainability [19]. Sago grubs are high in micronutrients (calcium, zinc, and iron) and have higher protein content compared to meat, dairy products, and grains [20]. The nutritional composition of sago grubs in Indonesia includes high protein content (10.39/100g), digestibility of 92%, high magnesium and zinc content [21], safe levels of heavy metals, and a fat content of 59.71%[22]. Analysis of the nutritional content of sago grub flour by Ariani (2018) showed that its protein content is 33.68 g/100g, twice as high as the protein in eggs (12.5 g/100g), and it is high in the amino acids glycine, phenylalanine, and lysine [23], [24].

Sago grubs have long been used as a medicine to maintain human health due to their beneficial compounds. Functional ingredients from sago grubs have been shown in many in vivo and in vitro studies to have gastrointestinal protection, antioxidant and anti-inflammatory activity, antibacterial activity, immunomodulatory effects, blood glucose and lipid regulation, hypotensive effects, and reduced risk of cardiovascular disease [25]. Given the research on the protein content of sago grubs and the benefits of protein in increasing hemoglobin (Hb) levels, this study will test sago grubs on mice and analyze their effectiveness in addressing anemia. In addition to protein, another food recommended for addressing anemia is food high in iron. One such food is moringa (Moringa oleifera L). Moringa leaves are a plant with benefits as a natural medicine [26]. They contain high levels of macronutrients and micronutrients that can minimize the risk of anemia [8]. According to research by Riansyah et al. (2021), moringa leaves contain chlorophyll, which gives them their green color, and have 25 times more iron than spinach [27]. Moringa leaves are a source of essential protein, vitamins, and minerals, including iron, calcium, and vitamin C [28]. In 100 grams of dried moringa leaves, there are 24.66–26.79 g of protein, 18.67–20.99 g of fiber, and 4.98-16.90 g of fat [29]. Additionally, moringa leaves are a valuable source of essential amino acids, supplying about 43% lysine, tryptophan, methionine, and cysteine, and are very rich in valine and leucine [28]. The iron concentration in 100g of dried moringa leaves is 97.9 mg, along with 17.6–39.6 mg of carotenoids, dietary fiber, vitamin B, vitamin C, calcium, and other essential nutrients, all with good bioavailability [30]. The vitamin C content in moringa leaves helps with more effective iron absorption [26].

Based on the background, sago grubs and moringa leaves are functional local foods that are high in protein and iron, with the potential to help manage anemia. Therefore, this study will analyze and prove whether the combination of sago grub flour and moringa leaf flour can increase hemoglobin levels, thus potentially being recommended for anemia treatment. To prove this, the combination of sago grub flour and moringa leaf flour will be administered to anemic mice induced with sodium nitrite.

2. Materials and methods

Tools and Material

The materials needed were a cabinet dryer, blender, 60 mesh flour sieve, sonde, mouse cage, Hb measuring device (EasyTouch) complete with strips, and a digital scale. The materials used were 30 BALB/c mice, sago grubs, standard feed in the form of AD II pellets, and moringa leaf flour. The experimental animals (mice) used had obtained ethical clearance through the research ethics committee of the Institute for Research and Community Service, Halu Oleo University, Number: 1132a/UN29.20.1.2/PG/2023.

Method

This research used a true experimental design with a pre- and post-test with control group design. The research was conducted at the Laboratory Animal Laboratory, Faculty of Medicine, Halu Oleo University, for 28 days (7 days of acclimatization and 21 days of intervention). The population in this study were anemic BALB/c mice induced with sodium nitrite (0.4 ml/day). The sample size used in this study was the minimum sample size according to WHO criteria, which is 5 mice (WHO, 2000). Anticipating dropout, 10% more mice were added, resulting in a total sample size of 30 mice. Each group consisted of 6 mice. After obtaining the Hb level measurement data of the mice, calculations were made using statistical analysis. The ANOVA test was used to determine the presence of strong changes with a significance level of $p<0.05$, followed by a post hoc test (LSD). The research was carried out in several stages:

1. Procedure for Making Sago Grub Flour
   The sago grubs were processed into flour according to the research conducted by Ariani et al (2018), with the following manufacturing process: The sago grubs were washed and dried in an oven at 100°C for 5 hours, then mashed with a blender and sieved using a 60 mesh flour sieve. Based on the results of this study, it is known that the moisture content obtained from sago grub flour is 1.00%, which is in accordance with the Indonesian National Standard, which has a maximum moisture content limit for wheat flour of 14.5% [31].
2. Dosage of Sao Grub Flour and Moringa Leaf Flour
   The dosage of sago grub flour in this study refers to the research by Lestari, et al. in 2021, which used a dose of 0.36 g/BW/day in rats [21]. To obtain the dose in mice, the rat dose was converted to a mouse dose, resulting in 50.4 mg/gBW of mice. For the dosage of moringa leaf flour, it refers to the research by Nur Aina Rahmania in 2018 with a dose of 20.8 mg/20gBW in mice [32].

3. Combination of Sago Grub Flour and Moringa Leaf
   The combination formula in this study refers to the research by Shabrina Aulia and Farida Eko (2022), where the study used comparisons of 25%, 50%, and 75%, so that in the combination of sago grub flour and moringa leaf flour, the following formulations were obtained [33]:
   - T1 (25% sago grub flour and 75% moringa leaf flour) 28.2 mg/gBW
   - T2 (50% sago grub flour and 50% moringa leaf flour) 35.6 mg/gBW
   - T3 (75% sago grub flour and 25% moringa leaf flour) 43 mg/gBW.
4. Intervention Procedure for the Combination of Sago Grub Flour and Moringa Leaf Flour
   a. Day 1 to Day 7
      On the first day, Hb levels were checked in all groups of mice is positive control (PC), negative control (NC), treantment 1 (T1), treatment 2 (T2), and treatment 3 (T3). This was to ensure that all mice before being induced with sodium nitrite had normal Hb levels and were not yet categorized as anemic. The PC group was given regular feed in the form of pellets at a dose of 4-8 g/day and water at 5-8 ml/day [34].
      The NC group was made anemic by administering sodium nitrite. The following formula was used [35]:
      - Calculation of Sodium Nitrite Dose 250 mg/kgBW (0.25 mg/kgBW)
        Dose = sodium nitrite dose x average BW
        = 0,25 x 23
        = 5,75 mg
        Na.nitrite needed = (15 ml/vp) x DP
        = (15/1) x 5,75 mg
        = 86,25 mg
        = 0,086 grams
      - Volume of Sodium Nitrite Administration
        = average BW/max BW) xVP
        = (23/30) x 1
        = 0,7 ml/gBW
   b. Day 7
      - Hb levels of the PC group mice were measured, followed by continued administration of regular feed.
      - The Hb levels of the NC group mice that had been made anemic with sodium nitrite induction were measured.
      - The Hb levels of the T1, T2, and T3 treatment groups were measured in mice that had experienced anemia with sodium nitrite induction.
      - Blood samples were taken through the mouse tail. The mouse was placed on a holder, then the tail was cleaned using cotton soaked in 70% alcohol and wiped with warm water to increase blood flow. The tip of the mouse's tail was cut with surgical scissors by about 1 mm and pressed while massaging the base of the tail with fingers to make the blood flow. The blood was collected in an EDTA tube to prevent coagulation [36]. The blood samples obtained were then measured for hemoglobin levels using EasyTouch.
   c. Day 7 to Day 21
      - The PC group continued to be given regular feed in the form of pellets at a dose of 4-8 g/day and water at 5-8 ml/day (hedrich, 2014).
      - The NC group was no longer given sodium nitrite but continued to be given regular feed in the form of pellets at a dose of 4-8 g/day and water at 5-8 ml/day (hedrich, 2014).
      - The T1, T2, and T3 treatment groups were given a solution mixture of 25% sago grub flour and 75% moringa leaf flour with a volume of 0.9 ml/gBW for T1, a solution mixture of 50% sago grub flour and 50% moringa leaf flour with a volume of 0.8 ml/gBW for T2, and a solution mixture of 75% sago grub flour and 25% moringa leaf flour with a volume of 0.8 ml/gBW for T3.
   d. Day 21

The Hb levels of the mice in the positive control group, negative control group, and treatment groups T1, T2, and T3 were measured as the final data obtained.

3. Results and Discussions

Based on the results of data analysis, the observations of Hb levels in the 5 groups of mice are given below. Table 1 shows that after the induction of sodium nitrite in the negative control group and the treatment groups (T1, T2, and T3), all mice developed anemia. The sodium nitrite dose given to each mouse was 0.7 ml/tail for seven days. After the induction, Hb levels were measured, and the average Hb levels decreased from 10.8 g/dL to 7.7 g/dL in the negative control group, from 12.4 g/dL to 6.3 g/dL in group T1, from 11.9 g/dL to 6.1 g/dL in group T2, and from 11.9 g/dL to 6.3 g/dL in group T3.

The decrease in Hb levels after sodium nitrite administration can occur because sodium nitrite ($NaNO_2$) causes an increase in Reactive Oxygen Species (ROS). When ROS enters the circulatory system, it causes oxidative stress, leading to erythrocyte hemolysis [37]. Nitrite entering the blood then oxidizes Fe^{2+} ions in hemoglobin, causing the blood to form Fe^{3+} ions, which subsequently become methemoglobin. If methemoglobin levels are too high [38], red blood cells will undergo hemolysis, reducing oxygen delivery to all body tissues and causing anemia [39]. According to research by Syahrul Ardiansyah et al. (2022), sodium nitrite was administered orally at a dose of 3 mg/200 g body weight of rats. Sodium nitrite was dissolved in 3 mL of distilled water and given for 15 days. After measurement on day 16, a decrease in Hb levels in rats was observed. This is consistent with the research conducted by Sri Widyastuti et al. (2023), where a decrease in Hb levels in mice was observed after administering 0.3 mL/day of sodium nitrite orally for 14 days. In this study, sodium nitrite was administered intraperitoneally to avoid absorption problems, as the active substance (sodium nitrite) directly enters the bloodstream [40]. Therefore, the administration of sodium nitrite in this study required a shorter time compared to the oral route, only 7 days.

Table 1: Hasil Pengukuran Kadar Hb Pada Kelompok Mencit Hb Level Measurements in Mice Groups

Group	Mice	Hb Level g/dL					
		Baseline (Day 1)	Average	After Sodium Nitrite Induction (Day 7)	Average	After Intervention (Day 21)	Average
Control Positive (PC)	1	12,6	12,5	14,9	13,4	16,2	16,12
	2	10,4		13,8		16,3	
	3	11,1		13,8		16,2	
	4	14,1		12,2		16,1	
	5	14,1		12,4		15,8	
Negative Control (NC)	1	11,2	10,8	7,8	7,7	4,2	4,92
	2	10,3		7,3		4,9	
	3	11,2		7,9		5,2	
	4	11,2		8,2		7,2	
	5	10,0		7,3		3,1	
Treatment 1 (T1)	1	13,5	12,4	6,2	6,3	11,2	11,16
	2	13,3		7,9		11,8	
	3	12,1		6,4		10,5	
	4	11,6		5,5		11,5	
	5	11,7		5,5		10,8	
Treatment 2 (T2)	1	12,1	11,9	4,5	6,1	14,2	14,58
	2	10,5		7,1		14,7	
	3	11,5		6,2		14,8	
	4	14		4,7		14,3	
	5	11,2		7,9		14,9	
Treatment 3 (T3)	1	10	11,9	4,5	6,3	15,3	15,36
	2	11,2		5,9		15,4	
	3	12,9		7,9		14,8	
	4	11,2		6,2		15,5	
	5	14,2		7,1		15,8	

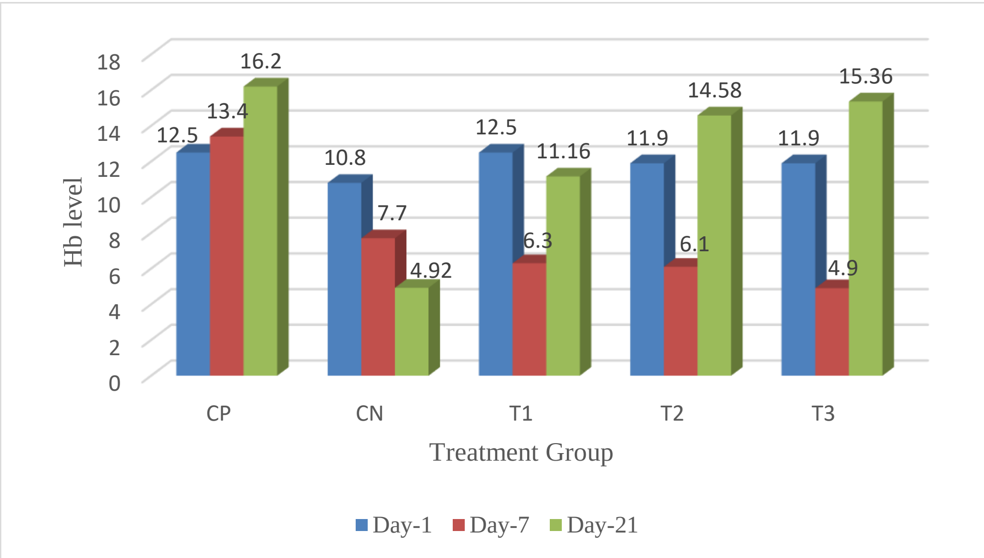

Figure 1: Hb Level Observations in Mice After Intervention

Table 2: One-Way ANOVA Results with Post Hoc Test (LSD) of Hb Level Data

Test Group	Comparison Group	Mean Diff	Sig.	Anova p-value
Control Positive	KN	112.00000**	.000	.000
	T1	49.60000**	.000	
	T2	15.40000**	.004	
	T3	7.60000*	.125	
Control Negative	CP	-112.00000**	.000	.000
	T1	-62.40000**	.000	
	T2	-96.60000**	.000	
	T3	-104.40000**	.000	
T1	CP	-49.60000**	.000	.000
	KN	62.40000**	.000	
	T2	-34.20000**	.000	
	T3	-42.00000**	.000	
T2	CP	-15.40000**	.004	.000
	KN	96.60000**	.000	
	T1	34.20000**	.000	
	T3	-7.80000*	.116	
T3	CP	-7.60000*	.125	.000
	KN	104.40000**	.000	
	T1	42.00000**	.000	
	T2	7.80000*	.116	

Keterangan: * There were no significant differences (p>0,05)
** There were significant differences (p<0,05)

After the mice developed anemia, they were given interventions using three formulations: formula 1 (T1) was 25% sago grub flour and 75% moringa leaf flour (28.2 mg/gBW), formula 2 (T2) was 50% sago grub flour and 50% moringa leaf flour (35.6 mg/gBW), and formula 3 (T3) was 75% sago grub flour and 25% moringa leaf flour (43 mg/gBW). The changes in Hb levels after the interventions can be seen in the figure 1. The intervention in groups T1, T2, and T3 involved administering different dosages of the sago grub flour and moringa leaf flour combination to each treatment group. The aim was to determine the effectiveness of increasing Hb levels and to analyze the most effective formula. After administering the formulations for 14 days, Hb levels were measured. The researchers found that the positive control group, which was given a normal diet, had an Hb level of 16.2 g/dL, indicating an increase in normal Hb levels from the beginning to the end of the study. This increase is attributed to the healthy and normal state of the mice. In line with this, the PC group of mice had their diet and water intake maintained, and according to Rizka Chibriyah (2018), a good quality diet and sufficient food intake influence Hb levels and optimal health [41]. In the NC group, after being induced with sodium nitrite for seven days, the average Hb level was 7.7 g/dL. Subsequently, from day 8 to day 21, only a normal diet was given. On day 21, the Hb level in the NC group averaged 4.92 g/dL. The results of this study indicate that red blood cells and Hb require a long time to return to normal if a diet high in protein and iron is not consumed. Efforts to address iron deficiency anemia, besides providing Fe tablet supplementation, can also be done by improving dietary patterns and consuming foods rich in iron and other nutrients such as vitamin C, which helps increase iron absorption in the body[42].

Research conducted by Rini et al. (2024) showed a significant relationship between iron intake and the incidence of anemia. The study found that samples with insufficient iron intake had a 7.895 times greater risk of developing anemia compared to samples with sufficient iron intake [43]. This is also consistent with research by Tateishi (2023), which showed a potential relationship between dietary macronutrient balance (protein and iron), anabolic status, and Hb levels [44]. Hemoglobin levels will increase in line with sufficient iron intake [42]. Food sources that have the potential to prevent and control iron deficiency anemia include sago grubs and moringa leaves.

In this study, it was found that all three formulations of the sago grub flour and moringa leaf flour combination had an effect on increasing Hb levels in mice. In group T1 (25% sago grub flour: 75% moringa leaf flour) with a dose of 0.92 ml/gBW, Hb levels increased from 6.3 g/dL to 11.16 g/dL. In group T2 (50% sago grub flour: 50% moringa leaf flour) with a dose of 0.71 ml/gBW, Hb levels increased from 6.1 g/dL to 14.58 g/dL. In group T3 (75% sago grub flour: 25% moringa leaf flour) with a dose of 0.71 ml/gBW, Hb levels increased from 4.9 g/dL to 15.36 g/dL. Although all three treatment groups showed an increase in Hb levels, group T1 was less effective in increasing Hb levels compared to groups T2 and T3, which was also statistically proven. This is consistent with research conducted by Florince Oyay et al. (2021), which found that administering sago grub flour to Wistar rats increased Hb levels from 8.92 g/dL to 13.18 g/dL [45]. Research by Kavle (2023) stated that the iron content in sago grubs is 23 mg/kg BW [46], indicating that consuming 0.4 to 0.5 kg/day of sago grubs would meet the recommended iron requirements of 8.7 mg/day (children), 14.8 mg/day (teenage girls), 11.3 mg/day (teenage boys), and 14.8 mg/day (adult women) and 8.7 mg/day (adult men) [47], as part of a balanced diet. Therefore, sago grubs have great potential as a 'high' iron source for diets [46].

This study combined moringa leaves as a source of non-heme iron with sago grub flour. Moringa leaves are a local plant rich in nutrients. According to the Indonesian Food Composition Table (IFCT), moringa leaves contain 7% iron per 100 g [48]. Combining iron sources from animal and plant sources increases the absorption of plant-based (non-heme) iron, and the fiber content in non-heme iron aids in the digestion process. Based on surveys, leafy green vegetables, when consumed regularly, play a significant role in iron balance [49]. Moringa leaf flour is a source of non-heme iron and vitamin C [50]. Research by Kustiani (2024) found that moringa pudding contains more than 3 g/100 g of dietary fiber, as well as 30% iron and zinc [51]. Research on moringa leaf extract by Nurmalasari et al. (2021) found that the most effective dose of moringa leaf extract in mice was 450 mg/kg BW, which increased Hb levels from 17.4g/dL to 19.3 g/dL [52]. Based on previous research, it can be concluded that both sago grub flour and moringa leaf flour can increase Hb levels. The results of this study indicate that the less sago grub flour (animal protein source) given, the lower the increase in Hb levels in anemic mice. This is evident in group T1, with a dose of 25% sago grub flour and 75% moringa leaf flour, showing the lowest increase in Hb levels compared to groups T2 and T3, which was also statistically proven (Table 2). Through the statistical analysis using One-way ANOVA, the result obtained was $p = 0.000$, which means $p<0.05$, indicating a significant effect in all treatment groups. Therefore, further data analysis was conducted using the Post Hoc Test (LSD) to determine if there were differences in the effects among each treatment group (T1, T2, T3).Based on the Post Hoc test results in Table 2 above, it is known that the NC group showed a p-value of 0.000 ($p<0.05$) in the LSD analysis, meaning that there was a significant difference between the NC group and the PC, T1, T2, and T3 groups. When comparing T1 with PC, NC, T2, and T3, the LSD analysis resulted in $p = 0.000$ ($p<0.05$), signifying significant differences between T1 and the PC, NC, T2, and T3 groups.

For T2 compared to PC, the LSD analysis yielded $p = 0.004$ ($p<0.05$), indicating a significant difference between T2 and PC. Furthermore, when comparing T2 with NC and T1, the LSD analysis showed $p = 0.000$ ($p<0.05$), signifying significant differences between T2 and both CN and T1. However, the comparison between T2 and T3 resulted in $p = 0.116$ ($p>0.05$), meaning there was no significant difference between T2 and T3. In the case of T3 compared to CN and T1, the LSD analysis produced $p = 0.000$ ($p<0.05$), indicating significant differences between T3 and both CN and T1. However, when comparing T3 with CP, the result was $p = 0.125$ ($p>0.05$), signifying no significant difference between T3 and CP. Similarly, the comparison between T3 and T2 yielded $p = 0.116$ ($p>0.05$), indicating no significant difference between T3 and T2.

4. Conclusions

Based on the conducted research, it can be concluded that the combination of sago grub flour and moringa leaf flour (T1, T2, and T3) has an effect on increasing hemoglobin levels in mice. Based on the analysis results, among the three formulations, groups T2 and T3 were the most effective in increasing hemoglobin levels in anemic mice.

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