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Research Article

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Effect of Ethanol from Extract of Tapak Liman Leaves (Elephantopus scaber Linn.) on Hematopoiesis of Anemia Mice

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ABSTRACT

Background and Objective: Tapak Liman leaves have been long used traditionally for anemia. The dried powder form of Elephantropus scaber was extracted to produce ethanol. This study aimed to prove the hematopoietic effect of tapak liman leaf. Materials and Methods: The study was conducted for 28 days. The samples consisted of one non-anemic group and four anemic groups induced by 130 mg/kgBW chloramphenicol given every day for 14 days. In the last day, group 2, 3 and 4 were given the extract of tapak liman leaves for 10, 30 and 100 mg/kgBW; respectively. Group 5 (as a positive control) was given physiological NaCl 0.9%. The testing doses were given once a day for 14 days, and the effects were observed on day 1st , 14th, 21st, and 28th. Results: The parameters used in this study were erythrocyte, hemoglobin, reticulocyte and hematocrit counts in white mice that were analyzed by two-way ANOVA showing a significant value of ($p<0.05$). Conclusions: The extract of tapak liman leaves could increase hematopoietic activities; hence, it could be used as an alternative therapy for anemia.

Key words: *Elephantopus Scaber Linn, Erythrocyte, Haematopoiesis, Hematocrit, Hemoglobin, Reticulocyte.*

INTRODUCTION

Red blood cells (erythrocytes) contain hemoglobin that enable red blood cells to carry oxygen from the lungs and deliver it throughout the body [1]. Hemoglobin consists of Fe (iron), protoporphyrin, and globin (1/3 of the weight of Hb consists of Fe) [2]. Decreasing blood cells (decreased red blood cells, white blood cells, and platelets) is called as anemia. It is a condition where hemoglobin (Hb), hematocrit, and red blood cell count below the normal value [3]. Anemia is a nutritional problem that affects millions of people in developing countries, and remains a major challenge for human health [4].

In Indonesia, one of the traditional herbs used as a treatment for anemia and blood booster is *tapak liman* [5]. *Tapak liman (Elephantopus scaber* L.) is a grass plant that grows in many regions, with an altitude of 1200 meters above the sea level [6]. Traditionally, this plant has also been efficacious as analgesic, diuretic, astringent and antiemetic. The leaves have been used to treat bronchitis, measles, diarrhea, and tonic [7]. The results showed this plant has diuretic, anti-inflammatory, and anti-tumor, treating arthritis, antibacterial, and antidiabetic activities, and can reduce the levels of LDL (Low-Density Lipoprotein) in the experimental animals [8]. The chemicals contained in *tapak liman* plant (*Elephantopus scaber* L.) are sesquiterpene lactone, scabertopin, isochlorogenic acid A and B, epifriedelinol, lupeol, stigmasterol, triacontane-1- ol, dotriacontane-1-ol, lupeol acetate, deoxyelephantopin [9]. Refluxed *tapak liman* sites have high iron (Fe) concentrations and very low copper (Cu) concentrations [10].

Based on the description above, the present study was done to prove the hematopoietic activity of *tapak liman* leaves, especially the *Elephantopus scaber* L. Hematological parameters observed in this study were the number

of erythrocytes, reticulocytes, hemoglobin level, and hematocrit value of the blood of male white mice with anemia.

MATERIAL AND METHODS

Time and Place

The research was conducted in February-April 2018 at Laboratory of Pharmacy Research (isolation and characterization) and Laboratory Serology-Immunology (treatment), Faculty of Pharmacy Universitas Andalas.

Materials

The materials on this research consisted of Na CMC (Bratachem), aquadest (Bratachem), ethanol 70% (Bratachem), hayem solution (Gersik Sarana Tirta), *brilliant cresyl blue* 1% (Merck), Chloramphenicol (Sanbe).

Equipment

The tools used in this study included Hemoglobin test (Easy Touch GCHb®), test strip hemoglobin (Easy Touch GCHb®), microscope (Olympus®), microcapillary hematocrit pipette (Nesco), centrifuge hematocrit (Scilogex), erythrocyte pipette (Assistant), and hemocytometer (Assistant).

Animal experimentation

Twenty mice (*Mus muculus, Swiss webster strain)* 2-3 months-old with body mass 20-30g from Pharmacology Laboratory, Faculty of Pharmacy Universitas Andalas were used. 7 days were allowed for acclimatization and observation before the treatment began.

Extraction *Elephantopus scaber* Linn.

4 kg of fresh *Elephantopus scaber* Linn. was sliced into 2-3 mm slices, then dried in a greenhouse for 3 days, then in a 50°C oven for 3 days. They were then blended to produce 400g of powder which was placed in a dark macerator bottle with 4L of 70% ethanol solvent, soaked for three days, stirring occasionally. The mixture was then filtered with filter paper four times until it was clear. The residue was then evaporated *in vacuo* with a rotatory evaporator until a thick extract was obtained. The ethanol extract was examined organoleptically, and a *rendement* test was conducted. The moisture and ash content were determined, as was the TLC profile.

The Treatment of Mice

130 mg/ kgBW dose of chloramphenicol was given to each mice every day for 14 days. Chloramphenicol suppressed the proliferation and differentiation of erythrocytes reducing the erythrocyte count in the blood producing anemia. Samples consisted of one non-anemic group, and four anemic groups induced by 130 mg/kgBW chloramphenicol given every day for 14 days. In the last day, group 2, 3 and 4 were given the extract of *Tapak Liman* leaves for 10, 30 and 100 mg/kgBW; respectively. Group 5 (as a positive control) was given physiological NaCl 0.9%.

Erythrocyte Count

A rinsed pipette was used for Hayem solution, the tail of the mice was cut off, and the wound was cleaned with a cotton swab. 0.5 µl of the blood from the mice was suctioned into the pipette, and the tip of the pipette was cleaned with tissue. Sufficient Hayem solution was pipetted up after the blood to make a total of 101 µl. The filled pipette was shaken for 3 minutes, two drops were discarded, then the tip was placed on a glass slide and covered with a coverslip. After 2-3 minutes for the erythrocytes to settle, a count was made under a microscope at 400x enlargement [11, 12].

Reticulocyte Count

Blood and brilliant cresyl blue dyes were mixed with a ratio of 1:1 in a tube and set aside for 15 minutes for the dye to be absorbed by the blood cells. 1-2 drops were dried on a slide, then examined under a microscope at 100x. Reticulocytes contained blue granules/filaments while mature erythrocytes appeared as clear light blue disks. The ratio of reticulocytes to 1000 erythrocytes was counted [11, 12].

Hemoglobin Level

The hemoglobin level was calculated using a device measuring hemoglobin (*GHB Easy Touch* brand), hemoglobin meters were already installed on the strips. Blood hemoglobin was inserted into the strip, and after10 seconds, the examination results were observed on the monitor of *Easy Touch GCHb* tool.

Hematocrit Level

Mice' blood was pipetted into a microcapillary pipette until ¾ full, and one tip was stopped with wax. The tube was centrifuged (microhematocrit centrifuge) at 16000 rpm for 5 minutes. The Hematocrit Level was measured by comparing the height of the solid fraction with the height of the solution in the microcapillary pipette.

Data analysis

The results of this study were statistically processed with IBM SPSS Statistic 22. The data were tested using two-way ANOVA and Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Tapak liman (*Elephantropus scaber* Linn.) leaves in this research have been identified in the Herbarium of the Biology Faculty of Mathematics and Natural Science, Andalas University. The identification was carried out to ascertain the identity of the sample so that no error happened in the sample used. Based on the identification results, it was shown that the samples used were the plant *tapak liman* (*Elephantopus scaber* Linn.) from Asteraceae family which could be seen in Figure 1.

Figure 1. Photograph of plants *tapak liman* (*Elephantropus scaber* Linn.)

After the extraction process by the maceration method, the viscous extract was obtained with a yield of 3.3%. The results of the organoleptic inspection of the condensed extract looked blackish brown, odorless with a bitter taste. The dried shrinkage and the total ash content of the condensed extract were 8.67% and 2.62%. *Tapak liman* also showed containing the flavonoid content of 6.20%. The results of the organoleptic inspection, dried shrinkage, ash content and the content of flavonoid *tapak liman* leaf extract according to the monograph extracts were identified by the Food and Drug Administration [13]. In order to determine the KLT profile of the extract, a mixture of n-Hexane eluent: ethyl acetate: methanol (5:5:1) was used, and a comparative compound of deoxyelephantopin was used in order to obtain the value of Rf 0.68. KLT profile of the *tapak liman* leaf extracts can be seen in Figure 2 which shows that the extract contained compounds of used deoxyelephantopin because it had the same Rf value as Rf of deoxyelephantopin was 0.68.

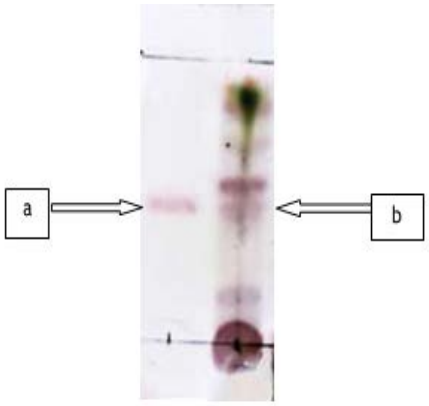

Figure 2. Profile of extract *tapak liman* KLT (*Elephantopus scaber* Linn.) Using silica gel F254 stationary phase and a mobile phase of ethyl acetate: n-Hexane: methanol (5:5:1) by *Lieberman Burcard* stain appearance.
Description: (a) = Deoxyelephantopin (comparison). (b) = Extract *tapak liman*.

In this study, chloramphenicol was used as an anemia inducer. Where chloramphenicol worked by suppressing the bone marrow to inhibit the reproduction and proliferation of bone marrow stem cells to any component of red blood cells that can cause aplastic anemia that can occur in a few days, weeks or even months depending on the size of the dose. Thus, the formation of erythrocytes components can be hampered and cause anemia. Anemia caused by chloramphenicol has been classified as aplastic anemia. Aplastic anemia is erythrocyte deficiency, reticulocyte, hemoglobin, and hematocrit as a result of the reduction of erythroblast cells produced in the bone marrow [14, 15]. 1000 mg/day doses of chloramphenicol were given which was converted in mice to 130 mg/kgBW. The dose given was also evidenced by the results of the orientation that has been done.

To determine the number of erythrocytes, the arithmetic room was used, and five particular fields of erythrocytes were considered. In the erythrocyte dilution pipette, a solution of Hayem was added with the aim to facilitate the calculation. Hayem solution could also contain lyse platelets and leukocytes that are visible only under a microscope. Erythrocytes looked like non-nucleated clear granules [16] which could be seen in Figure 3.

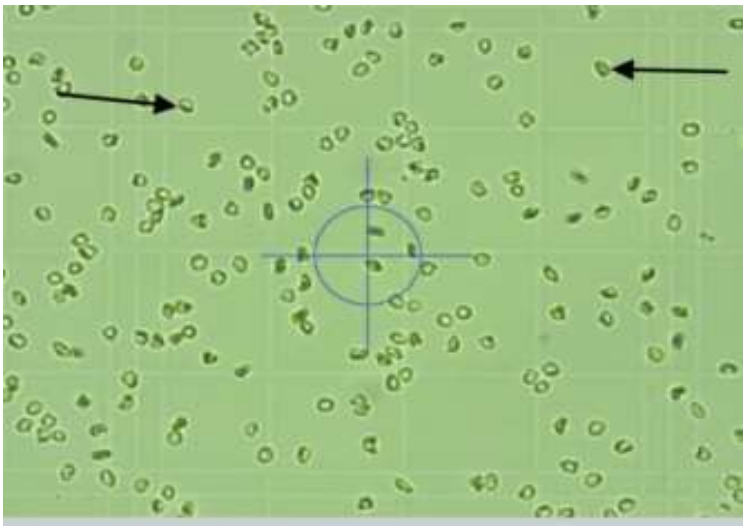

Figure 3. Photograph of male white mice erythrocytes using a counting chamber under a microscope magnifying 40 X.

Erythrocytes are the most abundant blood cells in the blood. After emerging from the bone marrow and into the bloodstream with a lifespan of about 120 days before disintegrating, they are replaced by new cells [17, 18]. Erythrocytes contain hemoglobin that enables red blood cells to carry oxygen from the lungs and deliver it to all body tissues [1]. Anemia, which is the lack of blood's ability to carry oxygen, occurs in mammals whenever the hemoglobin level drops below 12g/dl for women and 14g/dl for men. Individuals suffering from anemia also have a lower hematocrit level and reticulocyte count. The level of hematocrit is useful to diagnose the type of anemia, and the reticulocyte count indicates the condition of the bone marrow where Erythrocytes are produced.

The formation of erythrocytes started by hemocytoblast cells. Hemocytoblasts that were formed would differentiate and proliferate to be basophilic erythoblast. The process of hemoglobin formed started by phase basophilic erythrosit that differentiated erythroblasts polychromatophilic. These cells would differentiate again to be normoblast. After that, normoblast cytoplasm was filled with hemoglobin, the nuclei disappeared and reabsorbed by the endoplasmic reticulum of cells. These cells were then called reticulocytes because they contained the remains of the endoplasmic reticulum basophilic with hemoglobin in the cytoplasm. After the nuclei reticulum was reabsorbed, the cells became mature erythrocytes [19, 20]. The results of the calculation of the sheer number of mice erythrocytes anemia after treatment *tapak liman* leaf extract can be seen in Figure 4.

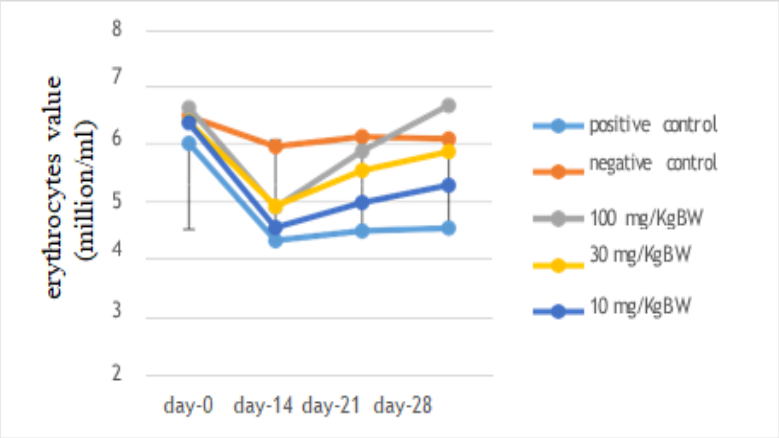

Figure 4. Graph of the relationship between the count of erythrocytes and the day of observation at each dose level after the *tapak liman* extract (*Elephantopus scaber* Linn.).

The results of two-way ANOVA test showed that the dose and the duration of the treatment with ethanol extract of *tapak liman* leave in anemia mice were significantly increasing the count of erythrocytes ($p<0.05$).

Table 1. Two-way ANOVA analysis of erythrocyte count after dosing with ethanol extract of *Elephantopus scaber* Linn.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	23.471	4	5.868	29.925	0.000
Duration	27.618	3	9.206	46.949	0.000
Doses and Duration	7.673	12	0.639	3.261	0.001
Total	58.762	19			

The results of Duncan's MRT test showed that the effects of each dose were significantly different ($p<0.01$), where the higher the dose applied, the higher erythrocyte could be produced. The erythrocytes count was significantly increased after the treatment with the extract of *tapak liman* leaves. The largest number of erythrocytes occurred in the third week. At that time, the dose of 100mg/kg could increase the number erythrocytes that were almost the same as normal mice ($p<0.01$). The effects of various doses were significantly different in a way that using the highest dose and conducting the longest treatment increased the activities of erythrocyte forming.

Table 2. DMRT analysis of erythrocyte count after dosing with ethanol extract of *Elephantopus scaber* Linn.

Treatments	N	Subset for alpha = 0.05			
		1	2	3	4
Doses					
Positive control	20	4.8390			
Negative control	20				6.1645
10mg/kgBW	20		5.2940		
30mg/kgBW	20			5.6785	
100mg/kgBW	20				6.0195
Sig.		1.000	1.000	1.000	0.304
Duration					
0^{th} day	25				6.780
14th day	25	4.9276			
21^{st} day	25		5.4016		
28^{th} day	25			5.6892	
Sig.		1.000	1.000	1.000	
		1.000			

To determine the reticulocyte count, a smear was made in advance, and followed by calculating the reticulocyte under the microscope. Reticulocyte cell looked like grains or a blue net with *brilliant cresyl blue* dye that were distinguishable from the colorless erythrocytes, as shown in Figure 5.

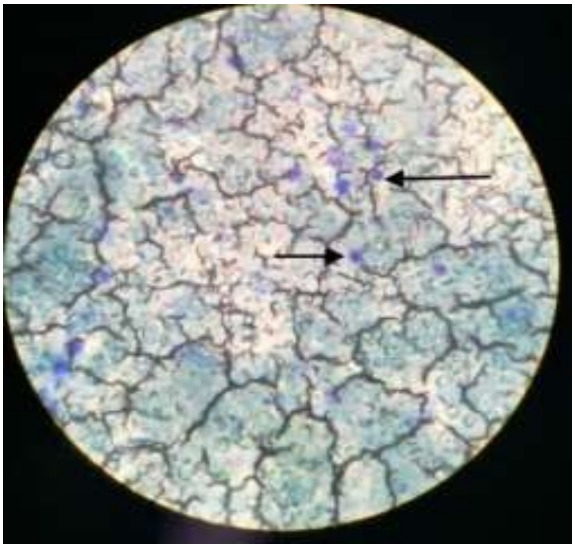

Figure 5. Reticulocyte cell photos of (a) the literature with 1000 x magnification, (b) blood smear white male mice after the treatment with the extract *tapak liman* (*Elephantopus scaber* Linn.) under a microscope with a magnification of 400x.

Reticulocyte count increased in all groups, as shown in Figure 6. Blood reticulocyte levels reflected a quantitative measure of erythropoietin, where erythropoietin stimulated erythroid stem cells to form the material for red blood cell formation [21]. Thus, the examination of reticulocyte had a clinical crucial role in helping the diagnosis of patients with anemia. Reticulocytes were young erythrocytes, which went into the capillary via diapedesis (squeeze through the pores of the membrane). After the reticulum was reabsorbed, all of the cells became mature erythrocytes. During the process of development, reticulocytes in the bone marrow made hemoglobin [19, 20, 22]. Therefore, when reticulocyte increased, the number of erythrocytes and hemoglobin concentration formed was also increased; this was consistent with the results obtained from the studies using two-way ANOVA stating that there was a significant effect of granting *tapak liman* leaf extract ($p<0.01$).

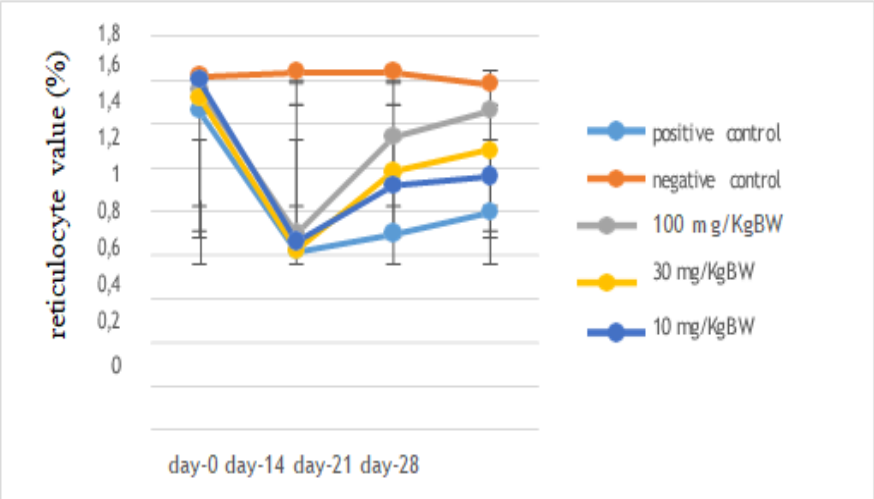

Figure 6. Graph of the relationship between reticulocyte counts and the observation days at each dose level after *tapak liman* leaf extract (*Elephantopus scaber* Linn.).

Table 3. Two-way ANOVA analysis of reticulocyte count after dosing with ethanol extract of *Elephantopus scaber* Linn.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	3.745	4	0.936	52.008	.000
Duration	3.749	3	1.250	69.428	.000
Doses and Duration	1.443	12	0.0118	6.682	.000
Total	8.937	19			

DMRT results showed that the doses of 10mg/kgBW and 30mg/kgBW were not significantly different in increasing the reticulocyte count ($p>0.05$), while the dose of 100 mg/kg was significant ($p<0.01$). That could be mean that the dose of 100 mg/kg increased reticulocytes to the greatest in anemia mice. The effect on the 21st day and 28th day showed that the number of reticulocytes was not significantly different ($p>0.05$). The increase in the number of reticulocytes in peripheral blood showed the increased production of erythrocytes in bone marrow. A low reticulocyte count indicated the bone marrow hypofunction or aplastic anemia [23].

Table 4. DMRT analysis of reticulocyte count after dosing with ethanol extract of *Elephantopus scaber* Linn.

Treatments	N	Subset for alpha = 0.05			
		1	2	3	4
Doses					
Positive control	20	1.045			
Negative control	20				1.620
10mg/kgBW	20		1.185		
30mg/kgBW	20		1.200		
100mg/kgBW	20			1.315	
Sig.		1.000	0.725	1.000	1.000
Duration					
0th day	25			1.552	
14th day	25	1.008			
21st day	25		1.236		
28th day	25		1.296		
Sig.		1.000	0.118	1.000	

The level of hemoglobin was performed using an *Easy Touch GCHb* which is a digital health tool and the newest product from *Nesco multi check* that served to measure hemoglobin. This tool is accurate, and also easy and convenient to use. The accuracy of this tool has been used as a benchmark standard in the measurement of hemoglobin as it approximated the actual results when compared with the other tools [12]. The results of applying ethanol extract of leaves *tapak liman* in mice anemia can be seen in Figure 7.

The results of the two-way ANOVA test showed that the levels of hemoglobin after the treatment with the extract of *tapak liman* leaves in all dose groups and the duration of the treatment significantly increased the level of hemoglobin in anemia mice ($p<0.05$).

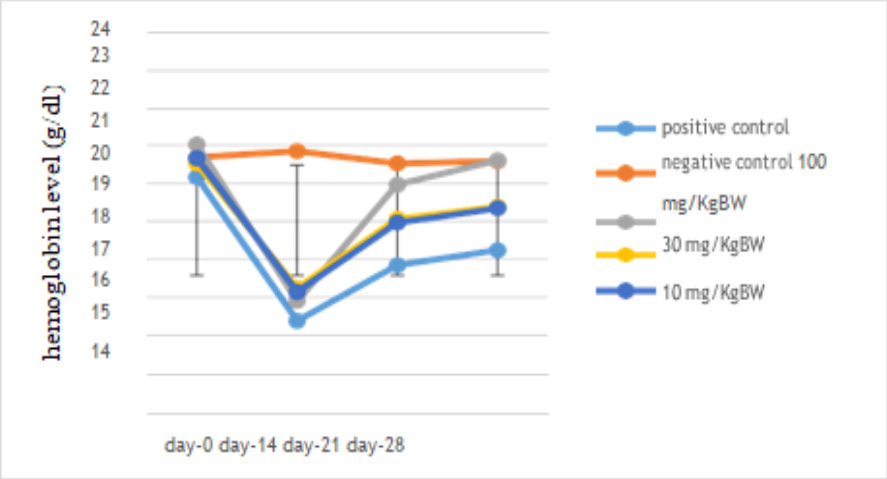

Figure 7. Graph of the relationship between hemoglobin levels and days of observation at each dose level after the extract of *tapak liman* (*Elephantopus scaber* Linn.)

Table 5. Two-way ANOVA analysis of Hemoglobin levels after dosing with ethanol extract of *Elephantopus scaber* Linn.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	67.751	4	16.938	53.882	0.000
Duration	109.487	3	36.496	116.099	0.000
Doses and Duration	38.744	12	3.229	10.271	0.000
Total	215.982	19			

The DMRT test results showed that hemoglobin levels in all dose groups were divided into 4 different groups. It was seen that the greater the dose, the bigger the effects of the amount of hemoglobin in mice anemia were. The level of hemoglobin in anemia mice at the dose of 10 and 30 mg was not significantly different ($p>0.05$). While the duration of the treatment was highly significant, and the level of hemoglobin increased in every week ($p<0.01$).

Table 6. DMRT analysis of hemoglobin levels after dosing with ethanol extract of *Elephantopus scaber* Linn.

Treatments	N	Subset for alpha = 0.05			
		1	2	3	4
Doses					
Positive control	20	18.155			
Negative control	20				20.655
10mg/kgBW	20		19.025		
30mg/kgBW	20		19.050		
100mg/kgBW	20			19.620	
Sig.		1.000	0.888	1.000	1.000
Duration					
0^{th} day	25				20.608
14^{th} day	25	17.700			
21^{st} day	25		19.268		
28^{th} day	25			19.628	
Sig.		1.000	1.000	1.000	

Hemoglobin is a substance that contains iron ions called hem (heme) and globulin proteins. There are about 300 of hemoglobins in the red blood cells. Hemoglobin serves to distribute oxygen from the lungs throughout the

body. Hemoglobin also carries carbon dioxide back to the lungs to be removed from the body [17].

The measurement of hematocrit level was conducted using the micro method. This method was selected because the amount of blood required was relatively small so that it could be used on the mice. When the mice was induced by chloramphenicol 130mg/kgBW, the hematocrit on mice decreased, and after the treatment by the extract of the leaves of *tapak liman*, the significant effect on increasing hematocrit in all groups of treatments ($p<0.05$) was observed. If the hematocrit level was low, the number of erythrocytes was low. Hematocrit level included the volume of all the erythrocytes in 100 ml of blood and was called the percent (%). The results of applying *tapak liman* leaf extract on mice anemia regarding hematocrit values can be seen in Figure 8.

The results of the two-way ANOVA showed the value of hematocrit after the treatment by *tapak liman* leaf extract against all dose groups, duration of treatment and interaction between the dose and duration of treatment which increased significantly ($p<0.05$).

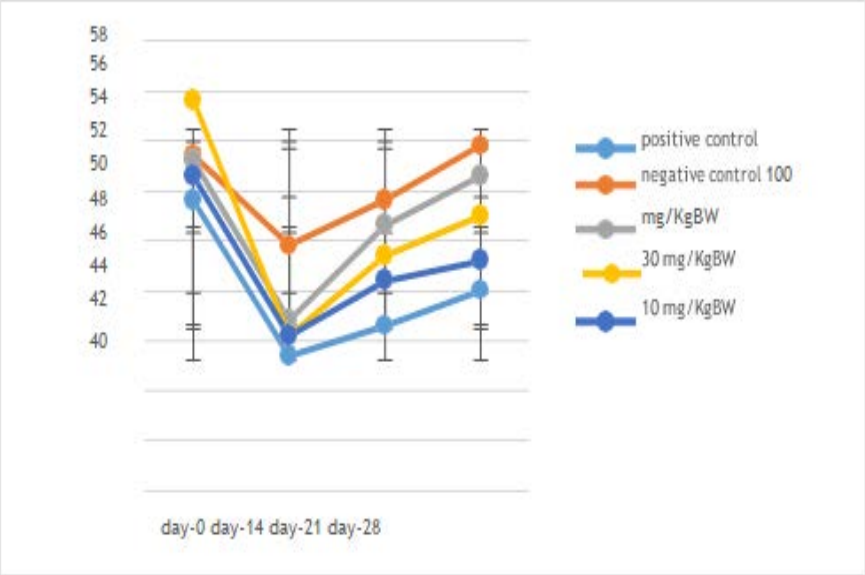

Figure 8. Graph of the relationship between hematocrit values of anemia mice and the time of extracting *tapak liman* leaves at each dose.

Table 7. Two-way ANOVA analysis of hematocrit level after dosing with ethanol extract of *Elephantopus scaber* Linn.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Doses	214.700	4	53.675	18.288	0.000
Duration	544.040	3	181.347	61.788	0.000
Doses and Duration	75.460	12	6.288	2.143	0.023
Total	834.200	60			

Based on the DMRT results and the hematocrit level, it was shown that the higher the dose, the greater the effect which could increase hematocrit in anemia mice. The increase in hematocrit level in doses of 30 and 100 mg/kgBW were really significant ($p<0.01$). The duration of treatment was highly significant ($p<0.01$) to increase the hematocrit level.

Table 8. DMRT analysis of hematocrit levels after dosing with ethanol extract of *Elephantopus scaber* Linn.

Treatments	N	Subset for alpha = 0.05			
		1	2	3	4
Doses					

Positive control	20	47.90			
Negative control	20				52.15
10mg/kgBW	20		49.10		
30mg/kgBW	20			50.55	
100mg/kgBW	20			50.80	
Sig.		1.000	0.888	1.000	1.000
Duration					
0^{th} day	25				53.28
14^{th} day	25	46.88			
21^{st} day	25		49.32		
28^{th} day	25			50.92	
Sig.		1.000	1.000	1.000	

Another research in anemia case with treatment by *Myrmecodia tuberosa* Jack, commonly known as *Sarang Semut* [20], showed that the ability of *Tapak Liman* leaves was better than *Myrmecodia tuberosa* Jack. It allegedly because deoxyelephantopin activities in *Tapak Liman* was greater than quercetin activities in *Myrmecodia tuberosa* Jack. to induce erythrocyte forming.

CONCLUSION

Based on the explanation above,it could be concluded that the doses of extract *tapak liman* leaf at 10mg/kgBW-100 mg/kgBW can increase the erythrocyte count, reticulocyte count, hemoglobin level, and hematocrit values. With the higher dose and longer duration of the treatment, the effects of the number of erythrocytes, reticulocytes, hemoglobin, and hematocrit anemia mice also got higher. It could mean that the extract *tapak liman* leaves have been very efective and efficient as a drug of anemia.

Significance Statement

This study discovered that the ethanol extract of *Elephantopus scaber* Linn. was able to increase the amount of erythrocyte and reticulocyte, the content of hemoglobin and the value of hematocrit in mice that can be beneficial as an effective treatment for many kinds of anemia. *Elephantopus scaber* Linn. grows abundantly in Padang. In isolated tropical areas, anemia due to hepatitis, pregnancies, and childbirth, malaria and kidney disorders have been significant problems. These are all anemias that could well be treated using an extract of *Elephantopus scaber* Linn. This study would help the researcher to uncover the critical areas of effectiveness of *Elephantopus scaber* Lnn.. against anemia. This plant could well become an economic and easily available treatment.

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