IJP Manuscript

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Submission date: 18-Dec-2021 04:46PM (UTC+0800)
Submission ID: 1733318959
File name: 89866-IJP-ANSI_Accepted.doc (803K)
Word count: 4425
Character count: 26339

Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack. in Anemic Mice

Short Title: (Effect of treatment of *Myrmecodia tuberosa* Jack. on anemic mice)

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ABSTRACT

Background and Objective: Previous research reported that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. increases phagocyte activity of macrophage, and lymphocyte proliferation and also prevents cutaneous anaphylactic reactions. Based on that, this present research aim to investigate the effect of an acetate fraction from *Myrmecodia tuberosa* Jack. on numbers of erythrocyte, reticulocyte, hemoglobin content and hematocrit in mice.

Materials and Methods: The research was conducted over 3 months and consisted of a positive control group and 3 groups treated with *Myrmecodia tuberosa* Jack. ethyl acetate fractions at 3 dosing levels. Anemia was induced in the mice using chloramphenicol 130 mg/kgBW for 14 days then for next 14 days daily oral doses of 40 mg/kgBW, 63.2 mg/kgBW or 100 mg/kgBW of *Myrmecodia tuberosa* Jack. ethyl acetate fraction were administered to each group. Blood samples were taken on day 0, 14, 21 and 28 for analysis. Statistical analysis was conducted using two-way ANOVA then Duncan Multiple Range Test (DMRT). **Results:** 40 mg/kgBW–63.2 mg/kgBW doses of *Myrmecodia tuberosa* Jack. ethyl acetate fraction significantly increased the erythrocyte, reticulocyte, and hemoglobin count and hematocrit from the 14th day ($p<0.01$). **Conclusions:** Ethyl acetate fraction of *Myrmecodia tuberosa* Jack. could have potential as an anemia treatment.

Keywords: Anemia, Erythrocyte, Hematocrit, Hemoglobin, *Myrmecodia tuberosa* Jack., Reticulocyte.

INTRODUCTION

Around 1,845 plants found in Indonesia are known to have been used as traditional medicine by different ethnic groups[1,2]. The shrubby caudex forming epiphyte *Myrmecodia sp*. is commonly called *Sarang semut,* literally ant nest plant, has been used medicinally in Papua, Mentawai Islands and Borneo. This genus contains a number of species with putative medicinal properties including *Myrmecodia tuberosa*, *Myrmecodia pendants* and *Hydnophytum formicarum* (*Rubiaceae*)[3]. They are known to contain flavonoids, triterpenoid, tocopherol, polyphenol, glycoside, tannin as well as calcium, sodium, calcium, zinc, iron, phosphorus and magnesium[4,5].

It has been found that the ethyl acetate fraction of *Myrmecodia tuberosa* can increase the phagocytic activity of macrophages and increase lymphocyte production in vitro[6]. These effects are thought to be related to the activity of phenol and flavonoid compounds. There is hope that by increasing lymphocyte cell proliferation these compounds could have anti-cancer properties [7] and could prevent active cutaneous nephrotoxic reactions [8]. The ethanol extract of *Myrmecodia tuberosa* has been found to increase SD (Sprague Dawley) mouse TCD4+ and TCD8+ *in vivo* after doxorubicin treatment[9]. *Myrmecodia tuberosa* flavonoids appear to have strong anti-inflammatory properties[10]. Terpenoid from *Myrmecodia tuberosa* has been found to have anti-cancer properties especially for human cervical cancer [11]. Flavonoids from *Myrmecodia tuberosa* killed a significant percentage of tongue cancer SP-C1 cells[12]. Also, a water extract of *Myrmecodia tuberosa* appears to cure diarrhea and improve bowel function[13].

When mature mammalian erythrocytes emerge from bone marrow they live about 120 days until disintegration and death. Dead erythrocytes are replaced by new cells which are produced by the bone marrow. White blood cells, unlike erythrocytes contain a nucleus and move independently. These are produced in the bone marrow and lymph nodes and play a role in eradicating disease[14].

Blood count is one indication of health status. Blood transports nutrients, oxygen, carbon dioxide, metabolites, hormones, antibodies and is essential in maintaining fluid balance and body pH[15].

Anemia results from lack of total blood or erythrocytes in the blood which hinders the transport of oxygen around the body. Erythrocytes contain the iron-containing complex protein hemoglobin. Anemia occurs when the hemoglobin level drops below 12 g/dl for a woman or 14 g/dl for a man. Low hematocrit value and reticulocyte count can also indicate the type of anemia present and the status of bone marrow, where erythrocytes are produced[16,17].

Anemia occurs frequently because of malnutrition leading to deficiency in iron, folic acid, or B_{12} but it can also be a result of damage to the stomach or compromised renal function leading to reduced erythropoietin production and infection. Anemia can also be a result of excessive breakdown and loss of erythrocytes due to heavy menstrual bleeding, childbirth, hemolysis or use of sustenance that irritate the stomach[18,19].

While anemia is a particular problem in isolated areas, low availability and lack of affordability put modern anemia medicines out of reach of those who most need it. Sometime the problem may be due to nutritional deficiencies but often what is needed is a way for the bone marrow to be stimulated to produce more erythrocytes. If components found in readily available and easy to cultivate plants can be found to achieve this aim then this could provide a solution to this problem.

Myrmecodia tuberosa Jack. already used traditionally to treat anemia, but no research has been conducted to determine its effectiveness or appropriate dose or duration of treatment. As flavonoids are thought to be the active ingredient in this plant in stimulating erythrocyte production, in this present research these were extracted from *Myrmecodia*

tuberosa Jack. using ethyl acetate and their effect on anemic mice investigated. Parameters measured were erythrocyte and reticulocyte counts, hemoglobin level and hematocrit value.

Material and Methods

Time and Place

The research was conducted in July-September 2017 at KOPERTIS Laboratory Region X, Pharmacy Research Laboratory Faculty of Pharmacy Universitas Andalas, and Serology-Immunology Laboratory of Faculty of Pharmacy Universitas Andalas.

Materials

The materials on this research consist of *Myrmecodia tuberosa* Jack. (Figure 1), ethyl acetate 1%; aquadest; ethanol 96%; Tween-80 0.1%; Carboxymethylcellulose (CMC) 0.1%; Drabkins Reagent (Catalog number: D5941 Sigma); Hayem solution (Catalog number: MFCD01866932 Sigma); cresyl blue brilliant 1%; and chloramphenicol 200mg/ml.

Equipment

Animal scales, maceration bottle, mortar, stamper, mice cage, measuring glass, sonde needles, thin-layer chromatography (TLC) plate, hematocrit pipette, hemoglobin pipette, Hettich centrifuge, *Uv-Visible* (BIO-RADx Mark) spectrophotometer, erythrocyte pipette, hemocytometer and microscope (ZEISS).

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 acclimatized and observation before treatment began.

Extraction and fractionation *Myrmecodia tuberosa* Jack.

4kg of fresh *Myrmecodia tuberosa* Jack. were sliced into 2-3 mm slices then dried in a greenhouse for 3 days then in a 50°C oven for 3 days. These 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 clear. The residue was then evaporated *in vacuo* with a rotatory evaporator until a thick extract was obtained[20,21].

This extract was dissolved in an equal volume of aquades and ethyl acetate solvent. The ethyl acetate fraction was pipetted off then evaporated until a viscous fraction remained. *Myrmecodia tuberosa* Jack.

Characterization of the Viscous Fraction

The viscous fraction of ethyl acetate was examined organoleptically and a *rendement* test conducted. The moisture and ash content was determined, as was the TLC profile.

Thin Layer Liquid Chromatography

A thin layer liquid chromatography profile of the ethyl acetate fraction was conducted using an eluent made from a mixture of butanol: acetate acid: water (2:0.5:2.5). The flavonol quercetin was used as a comparison.

The Treatment of Mice

130 mg/ kgBW dose of chloramphenicol was given to each mouse every day for 14 days. Chloramphenicol suppresses the proliferation and differentiation of erythrocytes reducing the erythrocyte count in the blood producing anemia[22]. The anemic mice were divided into four groups. The positive control group was orally dosed with a physiological saline solution and the second, third and fourth groups were given an oral daily 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW dose of the ethyl acetate fraction respectively.

Erythrocyte Count

A pipette rinsed was used Hayem solution, the tail of the mouse was cut off and the wound cleaned with a cotton swab. 0.5 µl of the blood from the mouse was suctioned into the

pipette and the tip of the pipette 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 discarded then the tip 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[19,23].

Reticulocyte Count

Blood and brilliant cresyl blue dye were mixed with ratio 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 contain blue granules/filaments while mature erythrocytes appear as clear light blue disks. The ratio of reticulocytes to 1000 erythrocytes was counted [19,23].

Hemoglobin Level

5 ml Drabkin solution was mixed with 20 µL blood and shaken in a tube until well mixed then set aside at room temperature for 3 minutes. Hemoglobin Level was determined using a spectrophotometer to measure absorbance at 546 nm [19,23].

Hematocrit Level

Mouse blood was pipetted into a microcapillary pipette until ¾ full and one tip 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

Correlations of these blood parameters with ethyl acetate dose were measured using two-way ANOVA. If significant correlations were found at the $p<0.05$ level these were further tested using DMRT (with IBM SPSS V20.0).

RESULTS AND DISCUSSION

The ethyl acetate fraction was viscous, aromatic, black-brown, and bitter. It contained 5.59% *rendement,* 11.44% moisture content and 6.24% ash. Results of the TLC indicate that only one major flavonol is present and it is, in fact, quercetin. The fraction had a retardation factor Rf of 0.78. The TLC profile can be seen in Figure 2.

Erythrocyte counts (million/µl) for 14 days of ethyl acetate fraction administration is shown in Table 1. The result of statistical analysis (Table 2) suggested that the erythrocyte count after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack. increased significantly ($p<0.01$) for all doses and durations of treatment. The increase in erythrocytes after 63.2 and 100 mg/kgBW doses were not significantly different at the $p<0.01$ level. The DMRT Test (Table 3) indicated that increasing dose size and duration of treatment significantly increases erythrocyte count ($p<0.01$); however, the difference between 63.2 mg/kgBW and 100 mg/kgBW doses was not significant.

Erythrocytes are the most numerous blood cells. There are many more erythrocytes compared to leukocytes and platelets. After emerging from the bone marrow where they are produced they live about 120 days before disintegrating and being replaced by new cells [14,26]. Erythrocytes contain hemoglobin which allows red blood cells to carry oxygen from the lungs and deliver it throughout the body tissues[25]. Anemia, lack of the ability of the blood to carry oxygen, occurs in mammals whenever hemoglobin level drops below 12 g/dl for female and 14 g/dl for male. Anemic individuals also have lower hematocrit levels and reticulocyte counts. Hematocrit levels are useful to diagnose the type of anemia and reticulocyte counts indicate the condition of the bone marrow where they are produced.

The used of chloramphenicol in this research served as an anemia inducer administered for 14 consecutive days. Chloramphenicol works to suppress the bone marrow so that it inhibits proliferation and differentiation. Thus, the formation of erythrocyte components can be inhibited and cause anemia. Anemia caused by chloramphenicol is

183 classified as aplastic anemia. Anema aplastic is a deficiency of erythrocytes, reticulocytes,
184 hemoglobin, and hematocrit as a result of reduction of erythroblast cells being produced in
185 the bone marrow[22,28].

186 Erythrocytes develop from hemocytoblast cells. New hemocytoblasts will continuously
187 form from bone marrow stem cells. Hemocytoblasts form basophilic erythroblasts which
188 begin to synthesize hemoglobin, and then erythroblast turns into polychromatophilic
189 erythroblasts, then the nuclei of these cells grow smaller and the cells produce hemoglobin
190 and become normoblast. After the cytoplasm of the normoblast is filled with hemoglobin, the
191 nuclei disappear and endoplasmic reticulum are reabsorbed by the cells. These cells are now
192 called reticulocytes because they still contain a few basophilic endoplasmic reticula which
193 stays with the hemoglobin inside the cytoplasm. The endoplasmic reticulum undergoes
194 capillary diapedesis, slipping out of the reticulocytes through membrane pores. After the
195 reticulum is all reabsorbed, cells become matured erythrocytes[16].

196 The reticulocyte count for 14 days of ethyl acetate fraction administration is shown in
197 Table 4. The increase is highly significant ($p<0.01$). The increase due to 40 mg/kgBW and
198 63.2 mg/kgBW and 100 mg/kgBW was highly significantly different ($p<0.01$).

199 The effect of ethyl acetate fraction dose and duration of treatment of reticulocyte is
200 shown in Table 5 and Table 6. The ethyl acetate fraction dose showed a similar relationship
201 with the reticulocyte count as it does with the erythrocyte count. This is to be expected as the
202 reticulocytes develop into erythrocytes so an increase in one implies an increase in the other.

203 The increase in reticulocyte count suggests that, as expected, chloramphenicol only
204 caused reversible suppression of the bone marrow function and did not permanently damage
205 its ability to produce erythropoietin[29]. On the contrary, the ethyl acetate fraction of
206 *Myrmecodia tuberosa* Jack. appears to stimulate reticulocyte production in the bone
207 marrow[28]. An increase in the number of reticulocytes in peripheral blood indicates increased

production of erythrocytes in the bone marrow. A low reticulocyte count would indicate bone marrow hypofunction or aplastic anemia[31,32].

The average content of hemoglobin (g/dl) for 14 days of ethyl acetate fraction administration is shown in Table 7. ANOVA analysis indicated a significant relationship between dose and duration on the hemoglobin level ($p<0.05$) (Table 8). Subsequent DMRT results (Table 9) showed while neither the 40 mg/kgBW or 63.2 mg/kgBW dose resulted in hemoglobin levels significantly higher than the positive control, the 100 mg/kgBW dose did result in a significant increase ($p<0.05$).

Hemoglobin carries iron ions called heme and globulin protein. There are around 300 hemoglobin in one erythrocyte. Hemoglobin carries oxygen from the lungs to other parts of the body and brings carbon dioxide back to the lungs where it is exhaled[14]. So the increase in hemoglobin due to the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. indicates an improved ability of the blood to transport oxygen.

Hematocrit values measured for 14 days of ethyl acetate fraction administration are shown in Table 10. There was a significant relationship between ethyl acetate fraction dose and duration of treatment (Table 11 and Table 12) and hematocrit value ($p<0.05$). Meanwhile, the interaction between doses of treatment and days of monitoring indicates there was no significant effect on the amount of hematocrit content ($P>0.05$). Thus, the effect caused by an ethyl acetate fraction of *Myrmecodia tuberosa* Jack. on the value of hematocrit value was highly significant.

Furthermore, this research can be continued to determine the activity of the active compounds in the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. by observing the cytokine and erythropoietin (EPO) production of cells under hypoxic conditions along with interleukin-1 (IL-1) and interleukin-9 (IL-9). These cytokine compounds are responsible for the proliferation and differentiation of stem cells into pronormoblasts then into erythrocytes. It is expected that the active compounds present in *Myrmecodia tuberosa* Jack. can support

some stages of the process of proliferation and differentiation in the process of erythrocyte formation and not affect other cells.

CONCLUSION

The conclusion of this research are the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at doses of 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW can increase the formation of erythrocytes in anemic mice. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., faster erythrocytes are produced. This suggests that *Myrmecodia tuberosa* Jack. has potential as an economic and effective source of treatment for some types of anemia.

Significance statement:

This study discover the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was able to increase the amount of erythrocyte, reticulocyte, the content of hemoglobin and value of hematocrit in mice that can be beneficial as an effective treatment for many anemias. *Myrmecodia tuberosa* Jack. grows abundantly in the Mentawai Islands. In isolated tropical areas, anemia due to hepatitis, pregnancies and childbirth, malaria and kidney disorders are significant problems. These are all anemias that could well be treated using an extract of *Myrmecodia tuberosa* Jack.. This study will help the researcher to uncover the critical areas of effectiveness of *Myrmecodia tuberosa* Jack. against anemia. This plant could well become an economic and easily available treatment.

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Figure 1. A fresh “ant nest” tuber *Myrmecodia tuberosa* Jack.

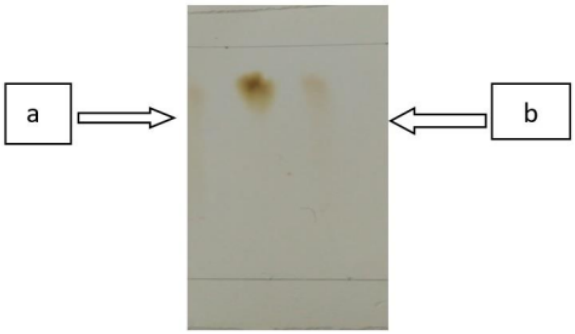

Figure 2. TLC profile of the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. under UV light (254 nm) using an eluent mixture of butanol: acetate acid: water (2:0.5:2.5). Note a = quercetin standard b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Table 1. Erythrocyte cells count in mice with anemia induced by 14 days of chloramphenicol and subsequent dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at different doses

Doses	Amount of erythrocyte (millions/µl)			average ± SD
	Day-14	Day-21	Day-28	
Positive Control	4.39±0.19	4.83±0.2	5.25±0.14	4.82±0.40
Dose 40 mg/KgBW	4.39±0.19	5.18±0.26	5.58±0.20	5.04±0.56
Dose 63.2 mg/KgBW	4.41±0.13	5.59±0.36	5.86±0.26	5.29± 0.70
Dose 100 mg/KgBW	4.45±0.15	5.61±0.08	5.99±0.09	5.35±0.69
Average ± SD	4.41±0.15	5.30±0.40	5.67±0.62	

Table 2. Two-way ANOVA analysis of erythrocyte count after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack..

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	2.638	3	.879	21.517	.000
Duration	16.927	2	8.464	207.115	.000
Doses and Duration	1.099	6	.183	4.483	.001
Total	1599.179	60			

Table 3. DMRT analysis of erythrocyte count after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Doses				
The positive control	20	4.8213		
40mg/kgBW	20		5.0447	
63.2mg/kgBW	20			5.2880
100mg/kgBW	20			5.3500
Sig.		1.000	1.000	.405
Duration				
14th day	20	4.4050		
21st day	20		5.3040	
28th day	20			5.6690
Sig.		1.000	1.000	1.000

Table 4. Reticulocyte count in mice with anemia induced by 14 days of chloramphenicol and subsequent dosing with ethyl acetate fraction of Myrmecodia tuberosa Jack. at different doses

Doses	Amount of reticulocyte (millions/µl)		average ± SD	
	Day-14	Day-21	Day-14	Day-21
Positive Control	0.42±0.04	0.68±0.08	0.78±0.04	0.63±0.17
Dose 40 mg/KgBW	0.48±0.08	0.76±0.11	0.86±0.09	0.70±0.19
Dose 63.2 mg/KgBW	0.44±0.05	0.78±0.08	0.96±0.11	0.73±0.24
Dose 100 mg/KgBW	0.42±0.08	1.02±0.15	1.38±0.13	0.94±0.43
Average ± SD	0.44±0.07	0.81±0.17	0.99±0.25	

Tabel 5. Two-way ANOVA analysis of reticulocyte count after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	.815	3	.272	30.191	.000
Duration	3.194	2	1.597	177.463	.000
Doses and Duration	.588	6	.098	10.895	.000
Total	38.630	60			

Table 6. DMRT analysis of reticulocyte count after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Doses				
The positive control	20	.6267		
40mg/kgBW	20		.7000	
63.2mg/kgBW	20		.7267	
100mg/kgBW	20			.9400
Sig.		1.000	.445	1.000
Duration				
14th day	20	.4400		
21st day	20		.8100	
28th day	20			.9950
Sig.		1.000	1.000	1.000

Table 7. Hemoglobin levels in mice with anemia induced by 14 days of chloramphenicol and subsequent dosing with ethyl acetate fraction of Myrmecodia tuberosa Jack. at different doses.

Doses	The content of hemoglobin (g/dl)			Average ± SD
	Day-14	Day-21	Day-28	
Positive Control	11.93±1.13	14.37±0.65	15.67±0.69	13.99±1.79
Dose 40 mg/KgBW	11.98±0.55	14.96±0.58	15.77±0.65	14.24±1.77
Dose 63.2 mg/KgBW	12.10±0.59	15.02±1.47	15.91±1.78	14.34±2.12
Dose 100 mg/KgBW	12.14±0.33	17.06±1.40	18.20±1.81	15.80±2.99
Average ± SD	12.04±0.66	15.35±1.46	16.39±1.65	

Table 8. Two-way ANOVA analysis of Hemoglobin levels after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Doses	30.103	3	10.034	8.403	.000
Duration	206.559	2	103.279	86.484	.000
Doses and Duration	12.834	6	2.139	1.791	.121
Total	13082.989	60			

Table 9. DMRT analysis of hemoglobin levels after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Doses				
The positive control	20	13.9887		
40mg/kgBW	20	14.2380		
63,2mg/kgBW	20	14.3440		
100mg/kgBW	20		15.7987	
Sig.		.407	1.000	
Duration				
14th day	20	12.0375		
21st day	20		15.3515	
28th day	20			16.3880
Sig.		1.000	1.000	1.000

Table 10. Hematocrit value in mice with anemia induced by 14 days of chloramphenicol and subsequent dosing with ethyl acetate fraction of Myrmecodia tuberosa Jack. at different doses.

Doses	Value of hematocrite (%)			Average ± SD
	Day-14	Day-21	Day-28	
Positive Control	41.9±1.75	44.2±1.48	45.6±1.48	43.9±2.09
Dose 40 mg/KgBW	43.8±2.49	45.0±1.58	46.9±2.22	45.2±2.37
Dose 63.2 mg/KgBW	43.2±2.59	45.2±1.95	47.4±1.14	453±2.56
Dose 100 mg/KgBW	43.5±2.57	46.7±2.73	49.2±2.92	46.5±3.5
Average ± SD	43.1±2.30	45.3±2.06	45.2±2.77	

Table 11. Two-way ANOVA analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Doses	49.483	3	16.494	3.622	.019
Duration	174.408	2	87.204	19.148	.000
Doses and Duration	10.692	6	1.782	.391	.881
Total	123126.000	60			

Table 12. DMRT analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
Doses				
The positive control	20	43.900		
40mg/kgBW	20	45.233	45.233	
63,2mg/kgBW	20	45.267	45.267	
100mg/kgBW	20		46.467	
Sig.		.103	.141	
Duration				
14th day	20	43.100		
21st day	20		45.275	
28th day	20			47.275
Sig.		1.000	1.000	1.000

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