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No.	Tanggal	Proses Kegiatan
1	5 Maret 2018	Submit artikel ke jurnal International Journal Pharmacology
2.	5 Maret 2018	Artikel diterima oleh pihak Jurnal IJP
3.	8 Maret 2018	Status artikel berubah dari "Assigned to Academic Editor" menjadi "Assigned to Scientific Review"
4	10 Maret 2018	Status artikel berubah menjadi "Revision Required for Manuscript Acceptance" –Revisi 1-
5	11 Maret 2018	Menyerahkan Revisi 1 pada pihak Jurnal melalui laman portal http://www.scialert.com/
6	16 Maret 2018	Status artikel berubah menjadi "Manuscript assigned to Reviewer for Scientific Review"
7	17 Maret 2018	Status artikel berubah menjadi "Revision Required for Manuscript Acceptance" –Revisi 2-
8	17 Maret 2018	Menyerahkan Revisi 2 pada pihak Jurnal melalui laman portal http://www.scialert.com/
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25	**19 Juli 2018**	**Pengiriman bukti pembayaran jurnal**
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27	**26 Agustus 2018**	***Galley proof* dikirimkan pihak Jurnal IJP**
28	**26 Agustus 2018**	**Balasan *galley proof* dikirimkan ke pihak jurnal IJP**
29	**3 September 2018**	**Status Artikel berganti menjadi published online first version**
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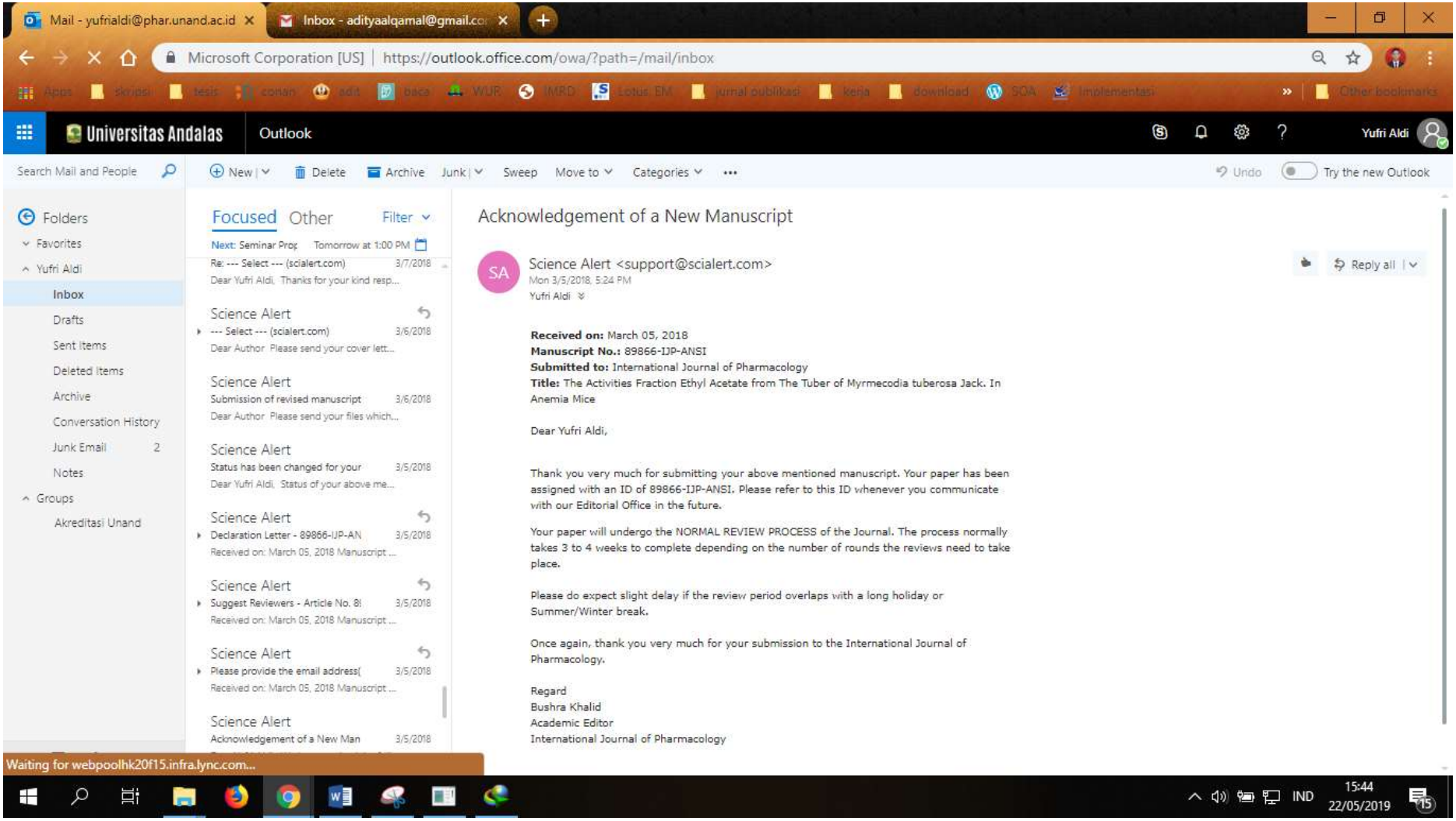

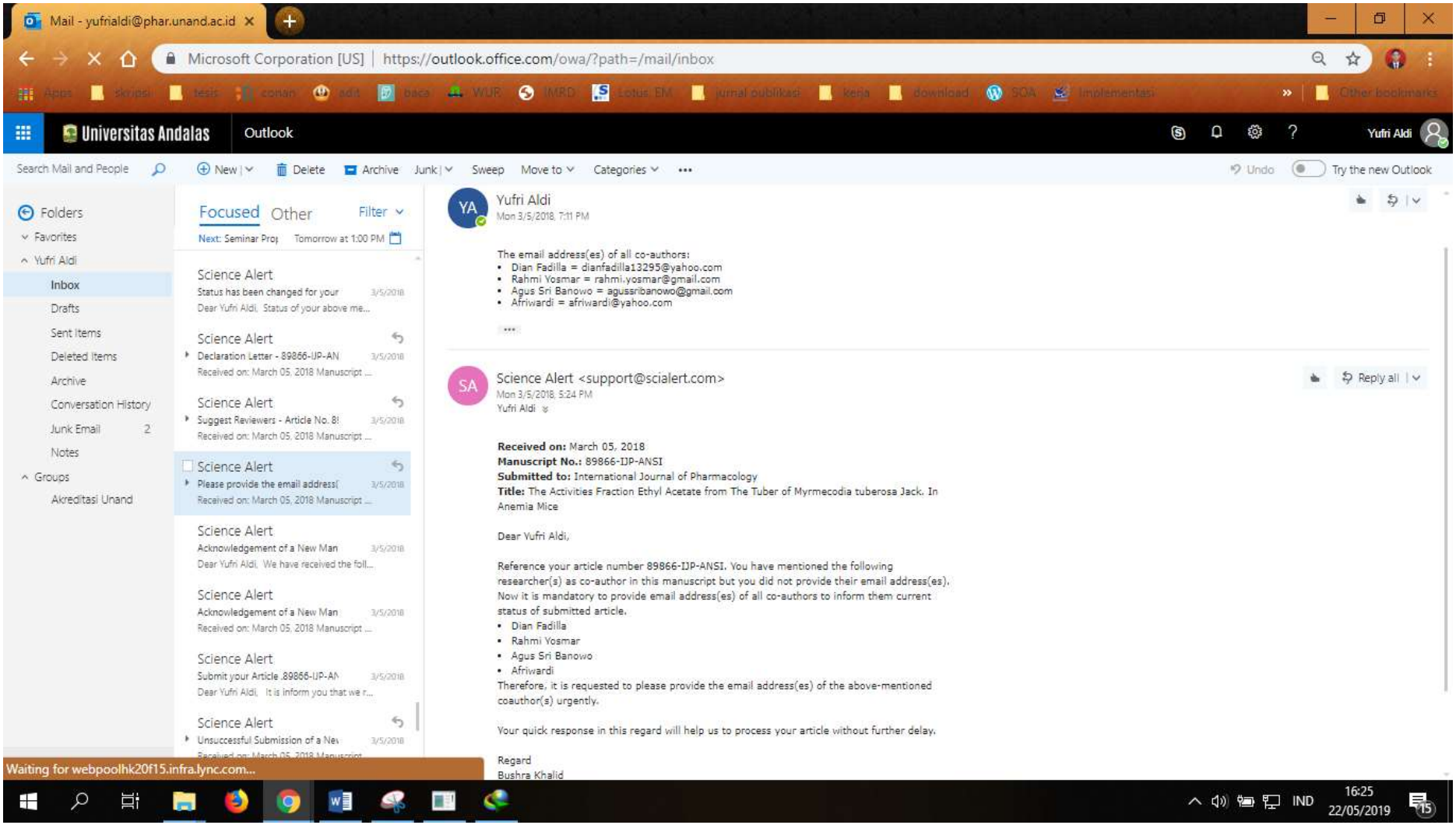
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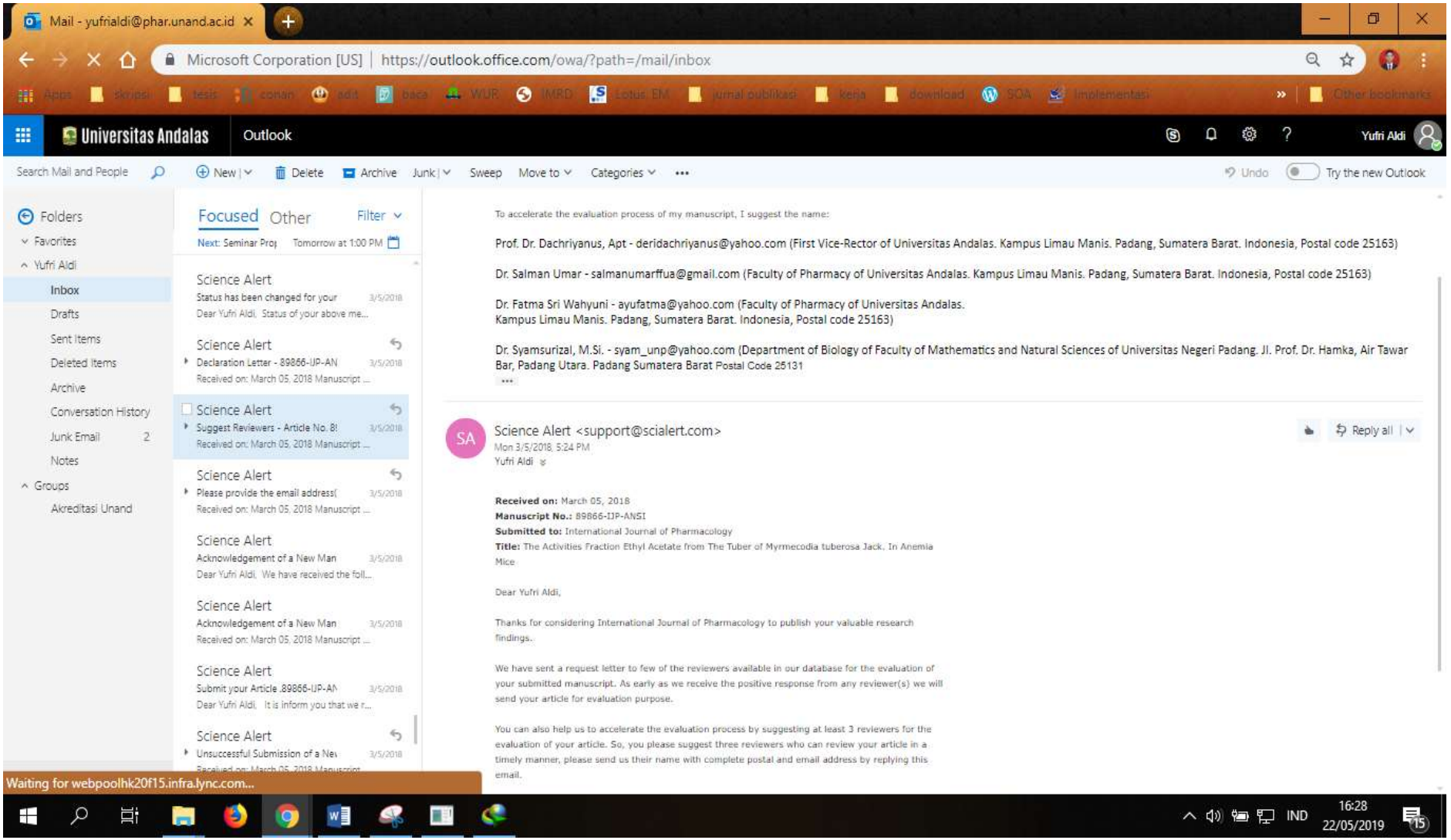
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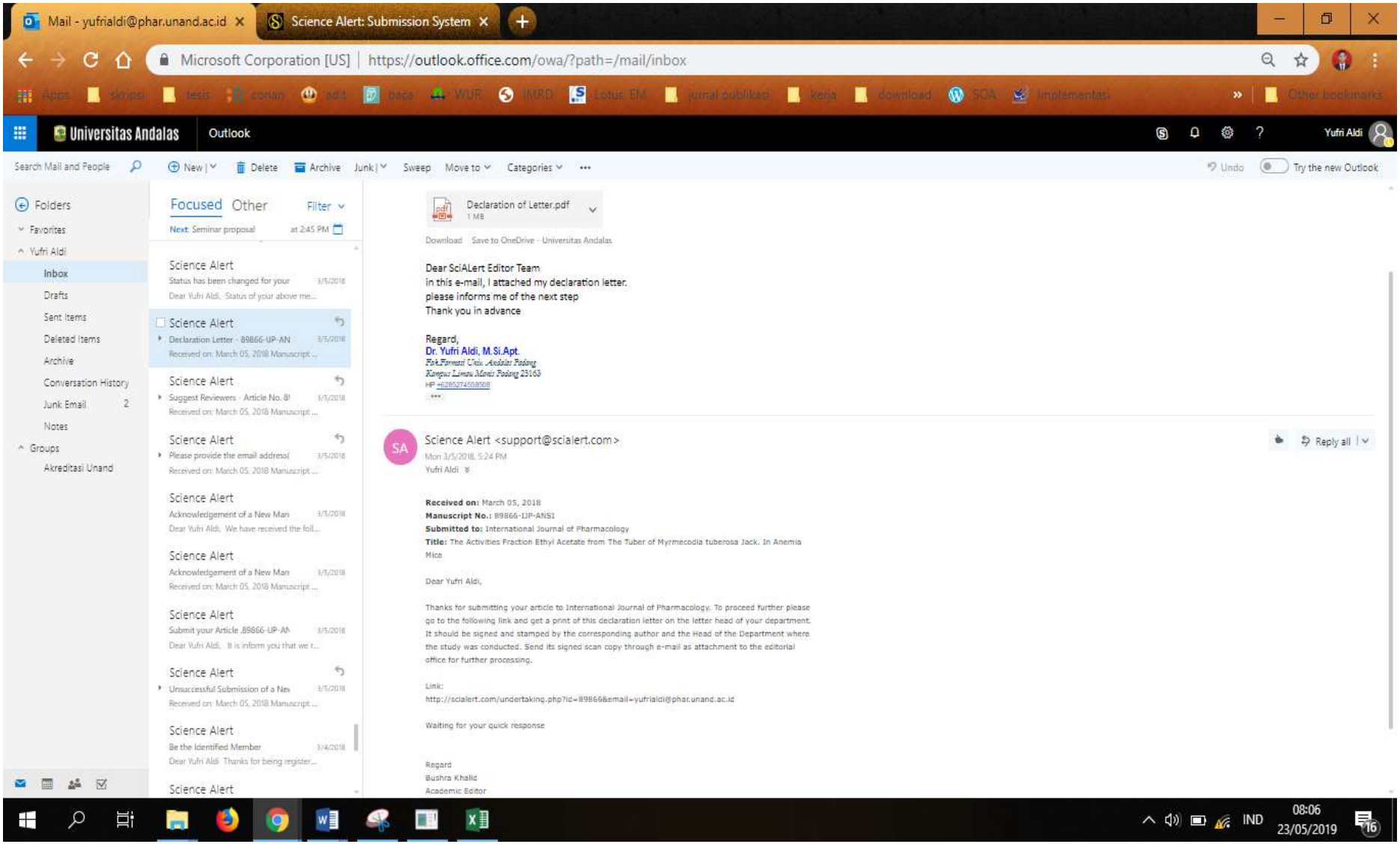
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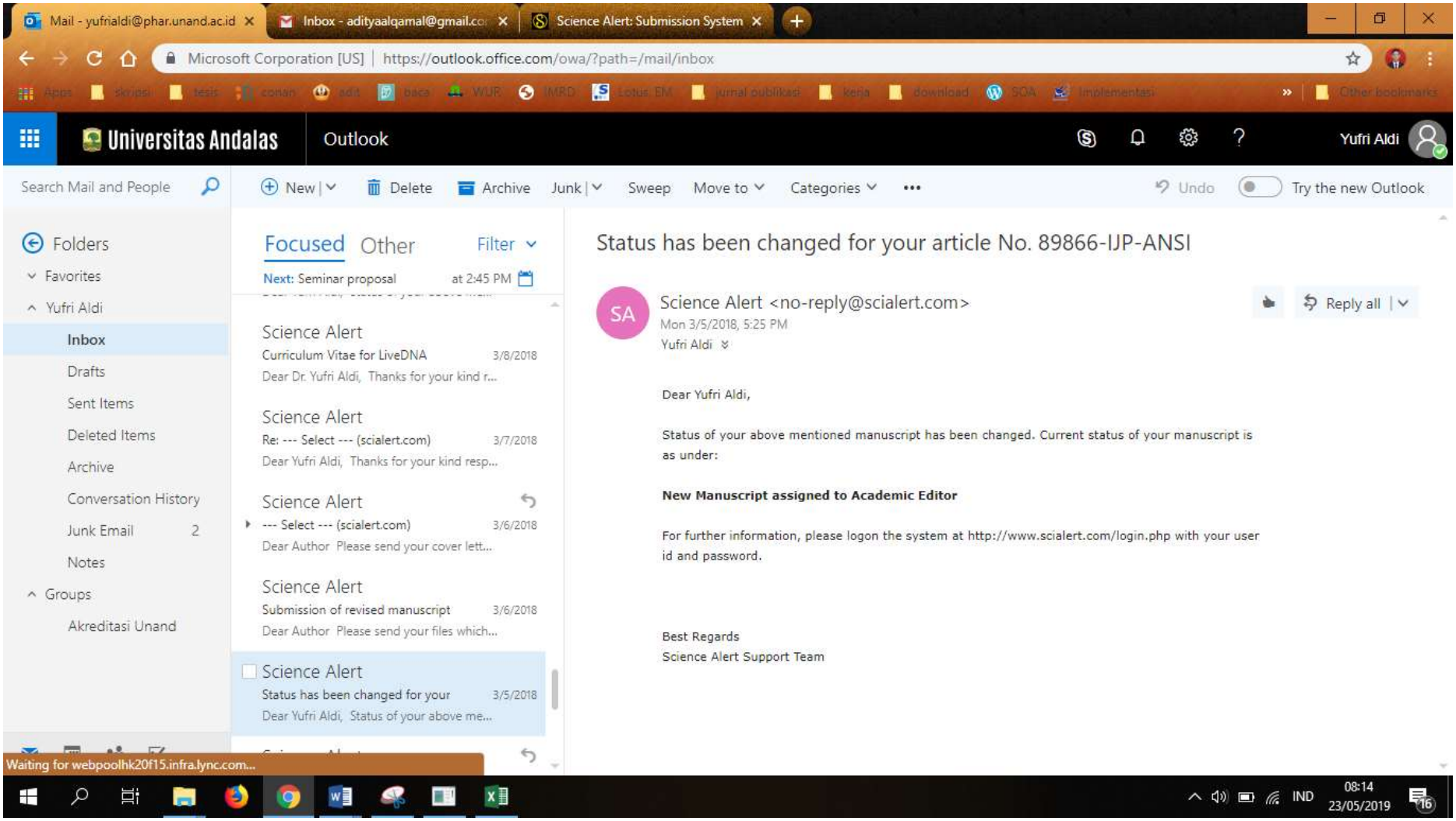

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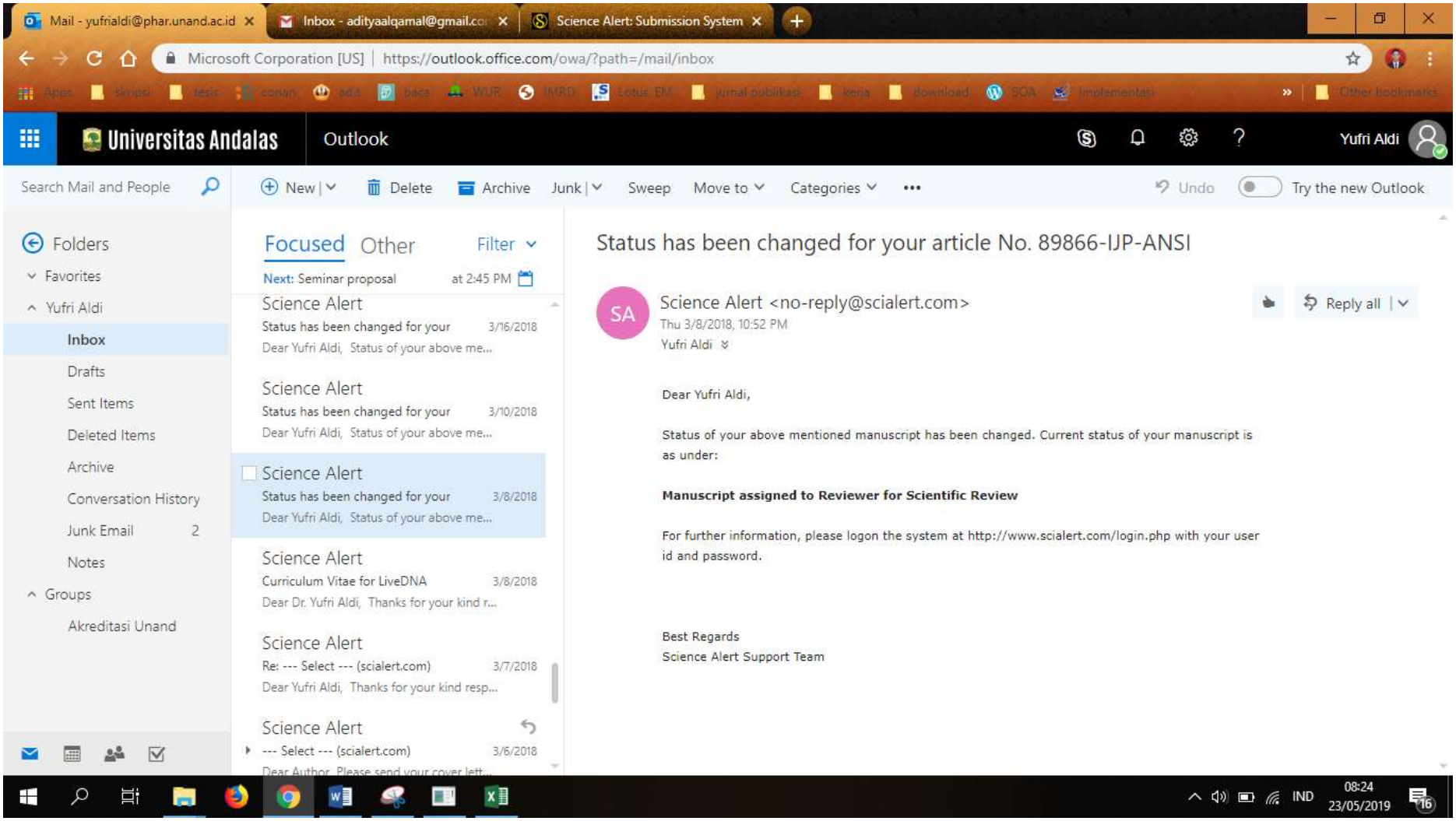

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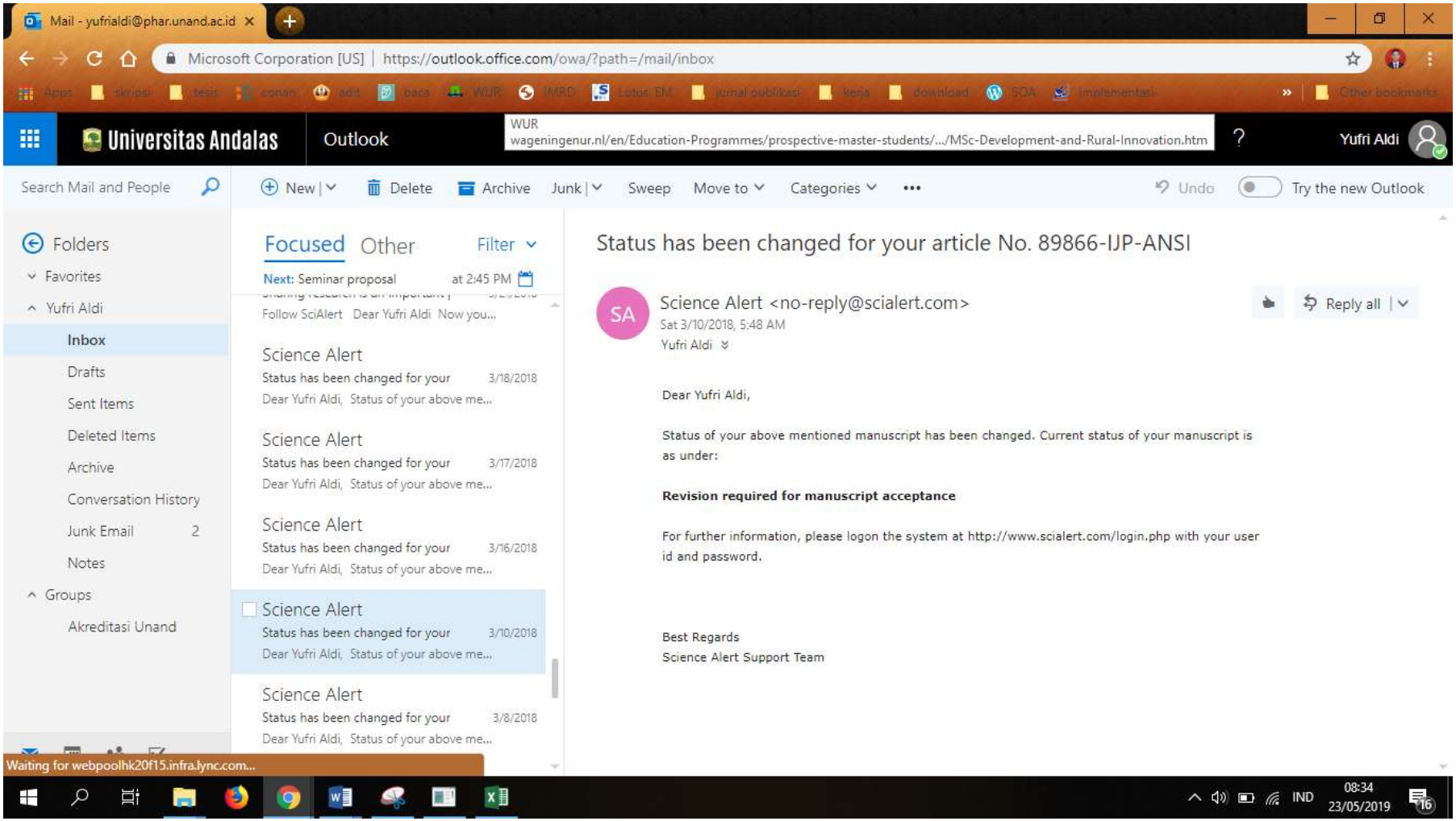

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- Abstract of the article must be structure into separate sections: Background and objective, the context and purpose of the study; Materials and Methods, how the study was performed and which statistical tests were being used; Results, the main findings; Conclusions, brief summary and potential implications of the study.
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- Briefly explain that how the powder of the *Myrmecodia tuberosa* Jack. was extracted?
- Statistical analysis section is not written properly. Mention the dependent and independent variable/ factors taken for the analysis of ANOVA. Also, provide the level of significance being used for the analysis.
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The Activities Ethyl Acetate Fraction of The *Myrmecodia tuberosa* Jack. In Anemia

(The Effect of treatment of *Myrmecodia tuberosa* Jack. on anemia mice)

Yufri Aldi[1]. Dian Fadilla[1], Rahmi Yosmar[1], Agus Sri Banowo[2], Afriwardi[3], Aditya Alqamal Alianta[4]

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ABSTRACT

Background and Objective: *Myrmecodia tuberosa* Jack has been used to increase the immunity and to counteract lack of blood during giving birth. Based on previous research, reported that the activities ethyl acetate fraction of *Myrmecodia tuberosa* Jack could improve the to increase phagocytes activity from macrophage and lymphocyte proliferation, also could prevent anaphylactic reaction of active cutaneous. Thus, based on that circumstance, the objective of this research was to continue the previous research to determine the influence of acetate fraction *Myrmecodia tuberosa* Jack. on blood cell formation by counting the amount of erythrocyte, reticulocyte, hemoglobin content as a parameter and the value of hematocrit on the mice. **Materials and Methods:** The research was conducted in 28 days and consists of a group control positive and 3 groups of treatment of an ethyl acetate fraction with 3 various doses. The mice were inducted with chloramphenicol 130 mg/kgBW for first 14 days in order to make them suffered anemia then for next 14 days it gave a fraction of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. orally with variation doses: 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW. Monitoring of mice condition was conducted on day 0, 14, 21 and 28. The data were analysed with two-direction ANOVA then analysed with DMRT **Results:** The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. significantly increaced the erytrocyte, retyculocyte, hemoglobin, hematocryte at 14th days with doses 40 mg/kgBW–63.2

mg/kgBW ($p<0.01$). **Conclusions:** The research showed that 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, hence it could be used as an alternative medicine for healing anemia.

Keywords: Erythrocyte, Hematocryte, Hemoglobin, *Myrmecodia tuberosa* Jack., Reticulocyte.

INTRODUCTION

Indonesia is in the second place after Brazil which has the highest biodiversity of the plant. Among of that, 1,845 plants were known as nitrous medicines that have been used as traditional medicine by many ethnics especially the society in villages on Indonesia[1,2].

One of the medicinal plants that very efficacious for the health was ant nest plant or usually called *Myrmecodia sp*. Commonly the tuber and nest plants that were used as the part of medication are *Myrmecodia tuberosa*, *Myrmecodia pendants* and *Hydnophytum formicarum* (*Rubiaceae*)[3]. It consists many chemical compounds such as flavonoids, triterpenoid, tocopherol, polyphenol, glycoside, tannin and also various minerals such as calcium, sodium, calcium, zinc, iron, phosphorus and magnesium[4,5].

The previous research showed the potential contents of ethyl acetate fraction of *Myrmecodia tuberosa* could increase phagocytes activity of macrophages and lymphocyte proliferation in vitro[6], it was related to phenol and flavonoids group compound that could increasing lymphocyte cell proliferation then became anti-cancer agent[7] and could prevent the reaction of active Cutaneous nephrotoxic[8]. Ethanol extract of *Myrmecodia tuberosa in vivo* could increase TCD4+ and TCD8+ in mice SD (Sprague Dawley) after they given doxorubicin[9]. Flavonoid of *Myrmecodia tuberosa* could decrease an inflammation with the most optimal anti-inflammatory power[10]. Terpenoid compound of *Myrmecodia tuberosa* was used as anti-cancer especially cervical cancer[11]. N-hexane fraction and water of *Myrmecodia tuberosa* ethanol extract showed significant percentage death of tongue cancer SP-C1[12]. Also, extract water of *Myrmecodia tuberosa* could healing diarrhea and increase feces consistency[13].

The Red blood cells mature is taken out by bone marrow and live about 120 days to have disintegration then die. Dead red blood cells are replaced by new cells which are produced by bone marrow. The White blood cells are different from erythrocytes because there are

nucleus and have independent movement. It made by bone marrow and lymph nodes to eradicate germs disease[14].

Blood depiction is one of concern in health status because blood is component that has important functions in maintaining the physiological body. In general, the function of blood was related to transport component in the body such as nutrient, oxygen, carbon dioxide, metabolites, hormone, head and immune body. Whereas, other function of blood-related to fluid balance and body pH[15].

Anemia was the condition lack of blood or red blood cell ability to carry oxygen that happened because of decreasing number of red blood cells. Anemia also happens when the hemoglobin level is under 12 g/dl in a woman and 14 g/dl in a man, diagnosis of anemia was not only observed by the numbers of erythrocytes and hemoglobin level but also hematocrit value and numbers of reticulocytes. Calculating hematocrit is important to find out types of anemia and it is used to find out spinal cord activities, where is the production place of red blood cells[16,17].

Based on that explanations, the research about ants' nest activity especially the species of *Myrmecodia tuberosa* Jack. toward red blood cells have not been conducted yet. Thus, this research was aim to determine the influence of ethanol extract fraction in *Myrmecodia tuberosa* Jack. activity toward component red blood cells formation with various observed in hematological parameters which are the number of erythrocytes, reticulocyte, hemoglobin level and hematocrit value that are done to mice that has an anemia.

RESEARCH METHOD

Materials

The materials on this research consist of *Myrmecodia tuberosa* Jack. (figure 1), ethyl acetate, aquadest, ethanol, Tween 80, Carboxymethylcellulose (CMC), Drabkins solution

(Sodium bicarbonate, potassium cyanide, potassium ferricyanide and aquadest), Hayem solution (sodium sulfate, sodium chloride, mercury chloride and aquadest) 1% of Cresyl blue brilliant and chloramphenicol.

Tools

The tools that were used animal scales, maceration bottle, mortar, stamper, mice cage, measuring glass, sonde needles, thin-layer chromatography (TLC) plate, hematocrit pipette, hemoglobin pipette, hettich centrifuge, spectrophotometer *Uv-Visible* (BIO-RADx Mark) erythrocytes pipette, hemocytometer and microscope (ZEISS).

Animal experimentation

The twenty of mice (*Mus muculus, Swiss webster strain)* was used which is 2-3 months old and weight about 20-30g (It is available in Pharmacology Laboratory, Faculty of Pharmacy Universitas Andalas). Before used, it was acclimatized for 7 days to observe animal behaviour.

Extraction and fractionation *Myrmecodia tuberosa* Jack.

The four kg of fresh *Myrmecodia tuberosa* Jack., slices into to 2-3 mm, then dried in a greenhouse for 3 days, and drying continued the oven at 50°C for 3 days. Thin slices of the *Myrmecodia tuberosa* Jack. has been dried and then blended, thus obtained 400g of *Myrmecodia tuberosa* Jack. powder. The powder of *Myrmecodia tuberosa* Jack. put it into dark macerator bottle, added 4L of 70% ethanol solvent. Its soaked for three days, stir occasionally and separate macerate by filtering with filter paper, repeat the filtering process for four times until the colour becomes clear. Collect all macerate then evaporated *in vacuo* with a rotatory evaporator until obtained a thick extract[18,19].

Thick extract of *Myrmecodia tuberosa* Jack. soluted with aquades and added ethyl acetate solvent with the same volume and insulated the ethyl acetate solvent then evaporated until obtained a congealed fraction of *Myrmecodia tuberosa* Jack.

The Congealed Fraction of Ethyl Acetate Characterization of *Myrmecodia tuberosa* Jack.

The congealed fraction of ethyl acetate was determined by organoleptic test, rendement test, a decrease of drying and ash, and the determination of its TLC profile

The Treatment of Animal Experimentation

To make the anemia mice, 130 mg/ kgBW dose of chloramphenicol was given every day for 14 days. Chloramphenicol gave a pressing spinal in order to hinder proliferation and differentiation so that the forming of red blood cells component was preventable and causing anemia[20] The anemia mice were divided into four group. The first group was positive control was given physiological NaCl solution then second, third and fourth groups were given a treatment of fraction of ethyl acetate of *Myrmecodia tuberosa Jack*. with each of the dose were 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW.

The Determination of Erythrocyte Number

Erythrocytes pipette at first was rinsed used Hayem solution, the tail of mice was cut off, clean the blood used a cotton and let the bleeding, then suction with pipette until the line of 0.5 µl, the blood that was in a tip of a pipette was cleaned with the tissue. Put the pipette in Hayem solution while holding the blood in line 0.5 µl, then it was sucked Hayem solution until the line of 101 µl. The pipette was lifted from solution, the cover of pipette tip using fingers and rubber suction tools was released. The filled pipette was shaken in 3 minutes, and first and second times drops were thrown away and the pipette tip was touched in surface counting space. Set aside the solution for 2-3 minutes in order erythrocyte settled. The number of erythrocytes was counted with the microscope with 400x enlargement[17,21].

Calculating Reticulocyte

Put the blood and dye (brilliant cresyl blue) into the tube with ratio 1:1, mix it well, set aside for 15 minutes so that the dye becomes perfect. Put the 1-2 drops of solution into blood film,

let it dry. Examined it under the microscope with the enlargement of 100x to make sure the solution has contained reticulocyte that showed by blue granules/filament and erythrocyte showed by light blue. Count the number of reticulocytes in 1000 cells erythrocyte [17,21].

Determination of Hemoglobin Level

Put 5 ml Drabkin solution into a tube. Took mice's blood from vein tail 20 µL, then put it in the tube. Shake the tube until 2 solutions were blended then set aside at room temperature for 3 minutes. Determine the absorbance value using spectrophotometer in the wavelength 546 nm[17,21].

Determination of Hematocrit Value

Took mice's blood then put it in pipette microcapillary until filled ¾ and one of a tip of the pipette was covered with a candle. Put microcapillary tube into centrifugation (microhematocrit centrifuge), then centrifuged at 16000 rpm for 5 minutes. Measure the height erythrocytes and the high of whole solution that is in microcapillary pipette.

Data Analysis

To determine the effect of a fraction of ethyl acetate and duration of treatment to erythrocyte value, reticulocyte, hemoglobin, and hematocrit were analysed with two-way ANOVA then continued with Duncan Multiple Range Test (DMRT).

RESULT AND DISCUSSION

The result of organoleptic indicated that the fraction was congealed, aromatic, the color was black-brown, and the taste was bitter. The ethyl acetat fraction obtained 5.59% rendement. The decrease of drying and ash content from ethyl acetate fraction of *Myrmecodia tuberosa* Jack. were 11.44% and 6.24%. To determine TLC profile of ethyl acetate fraction of *Myrmecodia tuberosa* Jack., eluen the mixture of butanol: acetate acid: water (2:0.5:2.5).

Signing compound which is used was quercetin. The obtained value of RF was 0,78 and its TLC profile could be seen in Figure 2.

Anemia was a condition which blood or red blood cells ability to carry oxygen was declining, usually caused by decreasing amount of circulating red blood cells. Anemia also occurs whenever the content level of hemoglobin was below 12 g/dl for female and 14 g/dl for male, it also showed from the value of hematocrit as well as an amount of reticulocyte in a low condition[22]. Calculating the value of hematocrit was necessary to find out types of anemia and calculating reticulocyte was useful to see activities of the spinal cord, in which red blood cell is produced.

To determine the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit was conducted on day 14, 21 and 28. In each group of mice, the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit could be seen after 14 days of giving chloramphenicol, then will increase on day-21 and 28 after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied.

Determining the average amount of erythrocyte (million/µl) was done after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied within 14 days and the result showed in Table 1.

The result of statistical analysis (Table 2) suggests that the amount of erythrocyte after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied to all doses groups, the duration of the application and interaction between doses and duration of application was increase significantly ($p<0.01$). The effect resulting from doses 63.2 and 100 mg/kgBW was the same as the increasing amount of erythrocyte ($P<0.01$). The result of DMRT Test (Table 3) showed that the duration of treatment significantly increase erythrocyte value ($p<0.01$) while the effect of each dose based on result DMRT Test (Table 4) was different significantly ($p<0.05$). The bigger doses and the longer treatment of the ethyl acetate fraction given, the

more visible the effects that occur, but the doses 63.2 mg/kgBW and 100 mg/kgBW was not significantly different.

The amount of reticulocyte after the application of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. within 14 days shown in Table 5. The result of statistical analyses (Table 6) showed that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. could increase the amount of reticulocyte significantly ($P<0.01$). The effect that caused by dose 40 mg/kgBW was the same as dose 63.2 mg/kgBW and dose 100 mg/kgWB was significantly different ($p<0.01$). The increasing of calculation reticulocyte suggest that nothing matters with the function of the spinal cord or erythropoietin stimulus[23]. This has proved that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was able to stimulate spinal in order to produce reticulocyte. The activity doses of ethyl acetate fraction and duration of treatment of reticulocyte could be seen in Table 7 and Table 8. It showed the same effect happened same as the effect on the number of erythrocytes above which mean the work of the compounds of ethyl acetate fraction to the erythrocytes and the reticulocytes is the same. Reticulocytes are young erythrocyte cells and will develop into erythrocytes.

The average content of hemoglobin (g/dl) determined after 14 days implementation of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. It could be seen in Table 9. The statistical analysis result (Table 10) showed that there was a significant effect of doses application as well as a period of monitoring (application length) on the content level of hemoglobin after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). Trough DMRT result (Table 11 and Table 12) it showed that the effect of dose 40 mg/kgBW and 63.2 mg/kgBW was not significantly different with the positive control, while to dose 100 mg/kgBW, was significantly increasing the value of hemoglobin ($p<0.05$). Hemoglobin was a substance that contains iron ion called hem (heme) and globulin protein. There are around 300 hemoglobin in one red blood cells. Hemoglobin functions to distribute oxygen from lungs to all over the body. Hemoglobin

also brings carbon dioxide back to the lungs to be blown out of the body[14]. So the differences the doses of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. to the increase of hemoglobin value was really significant.

Monitoring the value of hematocrit was conducted after applying an ethyl acetate fraction of *Myrmecodia tuberosa* Jack. for 14 days, it showed in Table 13. Statistical analysis (Table 14) indicates that there was a significant effect resulting from applying doses (Table 15) and duration of treatment (Table 16) on hematocrit value after applying ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). 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 really significant.

Erythrocyte contains hemoglobin which enables red blood cells to take oxygen from the lungs and distribute it through all over the network of body organs[14]. Mature red blood cells are taken out from the spinal cord and alive for around 120 days so that later undergo disintegration and die. The dead red blood cells were replaced by new cells which produced by a spinal cord[14]. Red blood cells are derived from hemocytoblast cells. New hemocytoblast will continuously form from cell primordial of a spinal cord. Hemocytoblast would form erythroblast basophilic which begin to synthesize hemoglobin, and then erythroblast turns into a form of erythroblast polychromatophilia, then to nucleus cells are getting decreased while hemoglobin was formed more in amount and cells change into a form of normoblast. After cytoplasm normoblast filled with nucleus hemoglobin, it becomes so tiny that it is discarded. At the same time, reticulum endoplasma were reabsorbed by cells, at this moment, cells are named reticulosis because they still contain a few reticulum endoplasma basophilic which stay with hemoglobin inside the cytoplasm. Cells in the stadium of reticulocyte enter capillary

diapedesis (slip into membrane pores). After reticulum is all reabsorbed, cells will become matured erythrocyte[16].

Based on the explanation above, 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, hence it could be used as an alternative medicine for healing anemia.

CONCLUSION

The conclusion of this research was:

a. The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at dose 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW can increase the forming of blood cells.
b. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., the more blood cells will produce.

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Figure 1. Picture of ant hill tuber *Myrmecodia tuberosa* Jack.

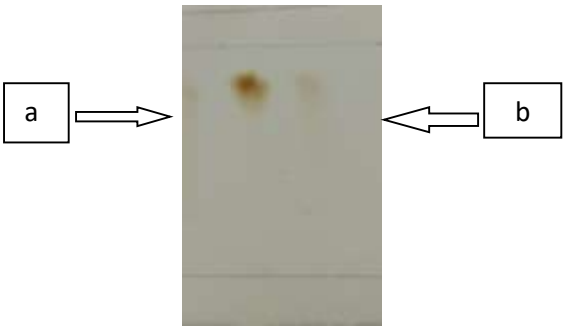


Figure 2. TLC profile of fraction (*Myrmecodia tuberosa* Jack.) below UV 254 nm with ethyl acetate solvent and an eluen mixture of butanol: acetate acid: water (2:0.5:2.5).
Note a = quercetin b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack

Table 1. The calculation result of the number of erythrocyte cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at a different doses within 14 days.

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. The result of two-way ANOVA analysis from erythrocyte value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis of treatment duration on erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14^{th} day	20	4.4050		
21^{st} day	20		5.3040	
28^{th} day	20			5.6690
Sig.		1.000	1.000	1.000

Table 4. The Result of DMRT analysis from erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05		
		1	2	3
The positive control	15	4.8213		
40mg/kgBW	15		5.0447	
63.2mg/kgBW	15			5.2880
100mg/kgBW	15			5.3500
Sig.		1.000	1.000	.405

Table 5. The calculation result of the number of reticulocytes cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at a different doses within 14 days.

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[a]
Dose 40 mg/KgBW	0,48±0,08	0,76±0,11	0,86±0,09	0,70±0,19[b]
Dose 63.2 mg/KgBW	0,44±0,05	0,78±0,08	0,96±0,11	0,73±0,24[b]
Dose 100 mg/KgBW	0,42±0,08	1, 02±0,15	1,38±0,13	0,94±0,43[c]
Average ± SD	0,44±0,07[a]	0,81±0,17[b]	0,99±0,25[c]	

Tabel 6. The result of two-way ANOVA analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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 7. The Result of DMRT analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05		
		1	2	3
The positive control	15	.6267		
40mg/kgBW	15		.7000	
63.2mg/kgBW	15		.7267	
100mg/kgBW	15			.9400
Sig.		1.000	.445	1.000

Table 8. The Result of DMRT analysis of treatment duration on reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	.4400		
21st day	20		.8100	
28th day	20			.9950
Sig.		1.000	1.000	1.000

Table 9. The result of determining the content level of hemoglobin of mice induced with chloramphenicol within 14 days, it continues with applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. tuber with different doses within 14 days.

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 10. The result of two-way ANOVA analysis from hemoglobin value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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 11. The Result of DMRT analysis from hemoglobin value after ethyl acetate fraction *of Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05	
		1	2
The positive control	15	13,9887	
40mg/kgBW	15	14,2380	
63,2mg/kgBW	15	14,3440	
100mg/kgBW	15		15,7987
Sig.		,407	1,000

Table 12. The Result of DMRT analysis of treatment duration on hemoglobin value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	12,0375		
21st day	20		15,3515	
28th day	20			16,3880
Sig.		1,000	1,000	1,000

Table 13. The result of determining hematocrit value of mice induced with chloramphenicol within 14 days, after applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. with different doses within 14 days.

Doses	Value of hematocrit (%)			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 14. The result of two-way ANOVA analysis from hematocrite value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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			

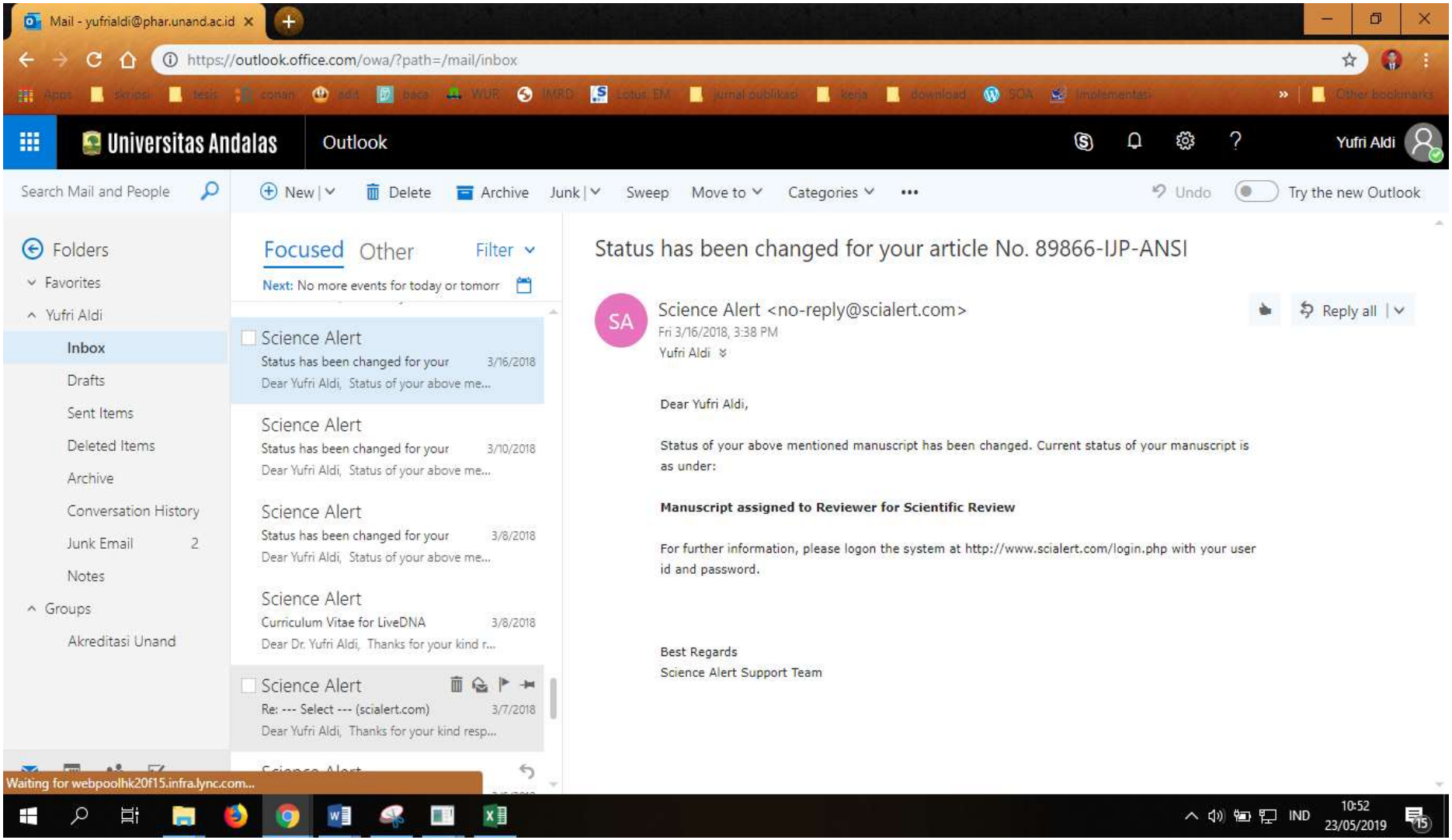
Table 15. The Result of DMRT analysis from hematocrite value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05	
		1	2
The positive control	15	43,900	
40mg/kgBW	15	45,233	45,233
63,2mg/kgBW	15	45,267	45,267
100mg/kgBW	15		46,467
Sig.		,103	,141

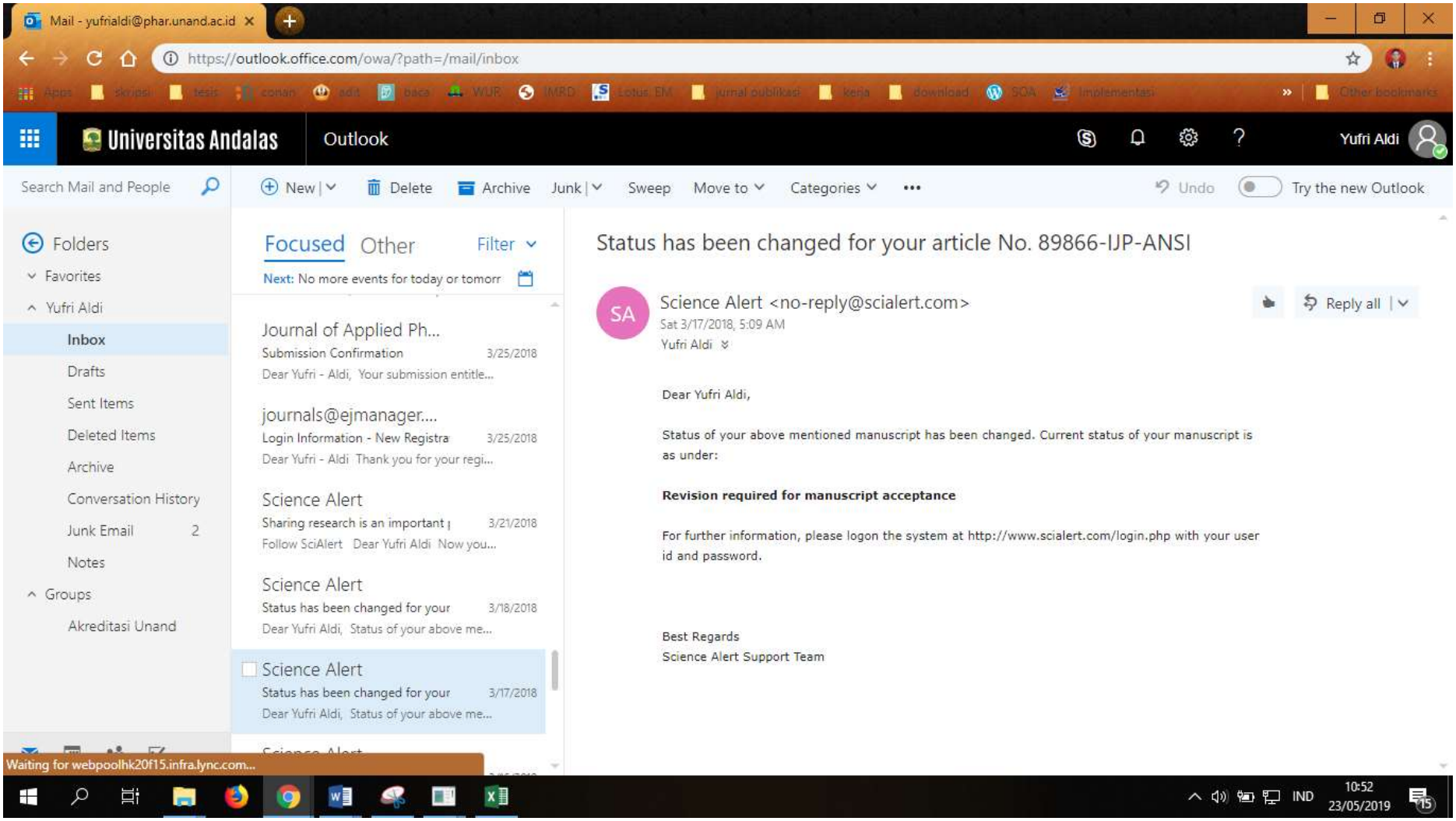
Table 16. The Result of DMRT analysis of treatment duration on hematocrite value after ethyl acetat fraction *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	43.100		
21st day	20		45.275	
28th day	20			47.275
Sig.		1.000	1.000	1.000

6. **Status artikel berubah menjadi "Manuscript assigned to Reviewer for Scientific Review"**



7. **Status artikel berubah menjadi "Revision Required for Manuscript Acceptance" –Revisi 2- (17 Maret 2018)**



8. **Menyerahkan Revisi 2 pada pihak Jurnal melalui laman portal http://www.scialert.com/**

ARTICLE NO: 89866-IJP-ANSI

Final Decision: Accepted After Major Revision

Reference your article entitled "The Activities Fraction Ethyl Acetate from The Tuber of Myrmecodia tuberosa Jack. In Anemia Mice" submitted for publication to International Journal of Pharmacology.

Revision that conducted:

- Structure of Abstract (Page 1)
- Information about the mice in this material and method (Page 5)
- The Methods how to get of the *Myrmecodia tuberosa* Jack. extracted (Page 5)
- Statistical analysis section is not written properly. Mention the dependent and independent variable/ factors taken for the analysis of ANOVA. Also, provide the level of significance being used for the analysis. (Result and Discussion)
- In tables and text of the article, a comma is present in the values. Is it really a comma or a dot? Kindly check it and update properly. (page 15)
- References with numbered, ordered sequentially as they appear in the text (Page 11-13)
- Short title/running title for the research paper that must be the punch line of the original title. (page 1)

The significance statement of novelty aspect and significance of this research work with respect to the existing literature and more generally to the society. (page 10/Conclusion)

The Activities Ethyl Acetate Fraction of The *Myrmecodia tuberosa* Jack. In Anemia

(The Effect of treatment of *Myrmecodia tuberosa* Jack. on anemia mice)

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ABSTRACT

Background and Objective: *Myrmecodia tuberosa* Jack has been used to increase the immunity and to counteract lack of blood during giving birth. It was reported that the activities ethyl acetate fraction of *Myrmecodia tuberosa* Jack could improve the to increase phagocytes activity from macrophage and lymphocyte proliferation, also could prevent anaphylactic reaction of active cutaneous. Thus, based on that circumstance, the objective of this research was to continue the previous research to determine the effect of acetate fraction *Myrmecodia tuberosa* Jack. on blood cell formation by counting the amount of erythrocyte, reticulocyte, hemoglobin content as a parameter and the value of hematocrit on the mice. **Materials and Methods:** The research was conducted in 28 days and consists of a group control positive and 3 groups of treatment of an ethyl acetate fraction with 3 various doses. The mice were inducted with chloramphenicol 130 mg/kgBW for first 14 days in order to make them suffered anemia then for next 14 days it gave a fraction of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. orally with variation doses: 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW. Monitoring of mice condition was conducted on day 0, 14, 21 and 28. The data were analysed with two-direction ANOVA then analysed with DMRT **Results:** The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. significantly increaced the erytrocyte, retyculocyte, hemoglobin, hematocryte at 14th days with doses 40 mg/kgBW–63.2 mg/kgBW ($p<0.01$).

Conclusions: The research showed that 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, hence it could be used as an alternative medicine for healing anemia.

Keywords: Erythrocyte, Hematocryte, Hemoglobin, *Myrmecodia tuberosa* Jack., Reticulocyte.

INTRODUCTION

Indonesia is in the second place after Brazil which has the highest biodiversity of the plant. Among of that, 1,845 plants were known as nitrous medicines that have been used as traditional medicine by many ethnics especially the society in villages on Indonesia[1,2].

One of the medicinal plants that very efficacious for the health was ant nest plant or usually called *Myrmecodia sp*. Commonly the tuber and nest plants that were used as the part of medication are *Myrmecodia tuberosa*, *Myrmecodia pendants* and *Hydnophytum formicarum* (*Rubiaceae*)[3]. It consists many chemical compounds such as flavonoids, triterpenoid, tocopherol, polyphenol, glycoside, tannin and also various minerals such as calcium, sodium, calcium, zinc, iron, phosphorus and magnesium[4,5].

The previous research showed the potential contents of ethyl acetate fraction of *Myrmecodia tuberosa* could increase phagocytes activity of macrophages and lymphocyte proliferation in vitro[6], it was related to phenol and flavonoids group compound that could increasing lymphocyte cell proliferation then became anti-cancer agent[7] and could prevent the reaction of active Cutaneous nephrotoxic[8]. Ethanol extract of *Myrmecodia tuberosa in vivo* could increase TCD4+ and TCD8+ in mice SD (Sprague Dawley) after they given doxorubicin[9]. Flavonoid of *Myrmecodia tuberosa* could decrease an inflammation with the most optimal anti-inflammatory power[10]. Terpenoid compound of *Myrmecodia tuberosa* was used as anti-cancer especially cervical cancer[11]. N-hexane fraction and water of *Myrmecodia tuberosa* ethanol extract showed significant percentage death of tongue cancer SP-C1[12]. Also, extract water of *Myrmecodia tuberosa* could healing diarrhea and increase feces consistency[13].

The Red blood cells mature is taken out by bone marrow and live about 120 days to have disintegration then die. Dead red blood cells are replaced by new cells which are produced by bone marrow. The White blood cells are different from erythrocytes because there are

nucleus and have independent movement. It made by bone marrow and lymph nodes to eradicate germs disease[14].

Blood depiction is one of concern in health status because blood is component that has important functions in maintaining the physiological body. In general, the function of blood was related to transport component in the body such as nutrient, oxygen, carbon dioxide, metabolites, hormone, head and immune body. Whereas, other function of blood-related to fluid balance and body pH[15].

Anemia was the condition lack of blood or red blood cell ability to carry oxygen that happened because of decreasing number of red blood cells. Anemia also happens when the hemoglobin level is under 12 g/dl in a woman and 14 g/dl in a man, diagnosis of anemia was not only observed by the numbers of erythrocytes and hemoglobin level but also hematocrit value and numbers of reticulocytes. Calculating hematocrit is important to find out types of anemia and it is used to find out spinal cord activities, where is the production place of red blood cells[16,17].

Based on that explanations, the research about ants' nest activity especially the species of *Myrmecodia tuberosa* Jack. toward red blood cells have not been conducted yet. Thus, this research was aim to determine the influence of ethanol extract fraction in *Myrmecodia tuberosa* Jack. activity toward component red blood cells formation with various observed in hematological parameters which are the number of erythrocytes, reticulocyte, hemoglobin level and hematocrit value that are done to mice that has an anemia.

RESEARCH METHOD

Materials

The materials on this research consist of *Myrmecodia tuberosa* Jack. (figure 1), ethyl acetate, aquadest, ethanol, Tween 80, Carboxymethylcellulose (CMC), Drabkins solution

(Sodium bicarbonate, potassium cyanide, potassium ferricyanide and aquadest), Hayem solution (sodium sulfate, sodium chloride, mercury chloride and aquadest) 1% of Cresyl blue brilliant and chloramphenicol.

Tools

The tools that were used animal scales, maceration bottle, mortar, stamper, mice cage, measuring glass, sonde needles, thin-layer chromatography (TLC) plate, hematocrit pipette, hemoglobin pipette, hettich centrifuge, spectrophotometer *Uv-Visible* (BIO-RADx Mark) erythrocytes pipette, hemocytometer and microscope (ZEISS).

Animal experimentation

The twenty of mice (*Mus muculus, Swiss webster strain)* was used which is 2-3 months old and weight about 20-30g (It is available in Pharmacology Laboratory, Faculty of Pharmacy Universitas Andalas). Before used, it was acclimatized for 7 days to observe animal behaviour.

Extraction and fractionation *Myrmecodia tuberosa* Jack.

The four kg of fresh *Myrmecodia tuberosa* Jack., slices into to 2-3 mm, then dried in a greenhouse for 3 days, and drying continued the oven at 50°C for 3 days. Thin slices of the *Myrmecodia tuberosa* Jack. has been dried and then blended, thus obtained 400g of *Myrmecodia tuberosa* Jack. powder. The powder of *Myrmecodia tuberosa* Jack. put it into dark macerator bottle, added 4L of 70% ethanol solvent. Its soaked for three days, stir occasionally and separate macerate by filtering with filter paper, repeat the filtering process for four times until the colour becomes clear. Collect all macerate then evaporated *in vacuo* with a rotatory evaporator until obtained a thick extract[18,19].

Thick extract of *Myrmecodia tuberosa* Jack. soluted with aquades and added ethyl acetate solvent with the same volume and insulated the ethyl acetate solvent then evaporated until obtained a congealed fraction of *Myrmecodia tuberosa* Jack.

The Congealed Fraction of Ethyl Acetate Characterization of *Myrmecodia tuberosa* Jack.

The congealed fraction of ethyl acetate was determined by organoleptic test, rendement test, a decrease of drying and ash, and the determination of its TLC profile

The Treatment of Animal Experimentation

To make the anemia mice, 130 mg/ kgBW dose of chloramphenicol was given every day for 14 days. Chloramphenicol gave a pressing spinal in order to hinder proliferation and differentiation so that the forming of red blood cells component was preventable and causing anemia[20] The anemia mice were divided into four group. The first group was positive control was given physiological NaCl solution then second, third and fourth groups were given a treatment of fraction of ethyl acetate of *Myrmecodia tuberosa Jack*. with each of the dose were 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW.

The Determination of Erythrocyte Number

Erythrocytes pipette at first was rinsed used Hayem solution, the tail of mice was cut off, clean the blood used a cotton and let the bleeding, then suction with pipette until the line of 0.5 µl, the blood that was in a tip of a pipette was cleaned with the tissue. Put the pipette in Hayem solution while holding the blood in line 0.5 µl, then it was sucked Hayem solution until the line of 101 µl. The pipette was lifted from solution, the cover of pipette tip using fingers and rubber suction tools was released. The filled pipette was shaken in 3 minutes, and first and second times drops were thrown away and the pipette tip was touched in surface counting space. Set aside the solution for 2-3 minutes in order erythrocyte settled. The number of erythrocytes was counted with the microscope with 400x enlargement[17,21].

Calculating Reticulocyte

Put the blood and dye (brilliant cresyl blue) into the tube with ratio 1:1, mix it well, set aside for 15 minutes so that the dye becomes perfect. Put the 1-2 drops of solution into blood film,

let it dry. Examined it under the microscope with the enlargement of 100x to make sure the solution has contained reticulocyte that showed by blue granules/filament and erythrocyte showed by light blue. Count the number of reticulocytes in 1000 cells erythrocyte [17,21].

Determination of Hemoglobin Level

Put 5 ml Drabkin solution into a tube. Took mice's blood from vein tail 20 µL, then put it in the tube. Shake the tube until 2 solutions were blended then set aside at room temperature for 3 minutes. Determine the absorbance value using spectrophotometer in the wavelength 546 nm[17,21].

Determination of Hematocrit Value

Took mice's blood then put it in pipette microcapillary until filled ¾ and one of a tip of the pipette was covered with a candle. Put microcapillary tube into centrifugation (microhematocrit centrifuge), then centrifuged at 16000 rpm for 5 minutes. Measure the height erythrocytes and the high of whole solution that is in microcapillary pipette.

Data Analysis

To determine the effect of a fraction of ethyl acetate and duration of treatment to erythrocyte value, reticulocyte, hemoglobin, and hematocrit were analysed with two-way ANOVA then continued with Duncan Multiple Range Test (DMRT).

RESULT AND DISCUSSION

The result of organoleptic indicated that the fraction was congealed, aromatic, the color was black-brown, and the taste was bitter. The ethyl acetat fraction obtained 5.59% rendement. The decrease of drying and ash content from ethyl acetate fraction of *Myrmecodia tuberosa* Jack. were 11.44% and 6.24%. To determine TLC profile of ethyl acetate fraction of *Myrmecodia tuberosa* Jack., eluen the mixture of butanol: acetate acid: water (2:0.5:2.5).

Signing compound which is used was quercetin. The obtained value of RF was 0,78 and its TLC profile could be seen in Figure 2.

Anemia was a condition which blood or red blood cells ability to carry oxygen was declining, usually caused by decreasing amount of circulating red blood cells. Anemia also occurs whenever the content level of hemoglobin was below 12 g/dl for female and 14 g/dl for male, it also showed from the value of hematocrit as well as an amount of reticulocyte in a low condition[22]. Calculating the value of hematocrit was necessary to find out types of anemia and calculating reticulocyte was useful to see activities of the spinal cord, in which red blood cell is produced.

To determine the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit was conducted on day 14, 21 and 28. In each group of mice, the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit could be seen after 14 days of giving chloramphenicol, then will increase on day-21 and 28 after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied.

Determining the average amount of erythrocyte (million/µl) was done after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied within 14 days and the result showed in Table 1.

The result of statistical analysis (Table 2) suggests that the amount of erythrocyte after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied to all doses groups, the duration of the application and interaction between doses and duration of application was increase significantly ($p<0.01$). The effect resulting from doses 63.2 and 100 mg/kgBW was the same as the increasing amount of erythrocyte ($P<0.01$). The result of DMRT Test (Table 3) showed that the duration of treatment significantly increase erythrocyte value ($p<0.01$) while the effect of each dose based on result DMRT Test (Table 4) was different significantly ($p<0.05$). The bigger doses and the longer treatment of the ethyl acetate fraction given, the

more visible the effects that occur, but the doses 63.2 mg/kgBW and 100 mg/kgBW was not significantly different.

The amount of reticulocyte after the application of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. within 14 days shown in Table 5. The result of statistical analyses (Table 6) showed that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. could increase the amount of reticulocyte significantly ($P<0.01$). The effect that caused by dose 40 mg/kgBW was the same as dose 63.2 mg/kgBW and dose 100 mg/kgWB was significantly different ($p<0.01$). The increasing of calculation reticulocyte suggest that nothing matters with the function of the spinal cord or erythropoietin stimulus[23]. This has proved that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was able to stimulate spinal in order to produce reticulocyte. The activity doses of ethyl acetate fraction and duration of treatment of reticulocyte could be seen in Table 7 and Table 8. It showed the same effect happened same as the effect on the number of erythrocytes above which mean the work of the compounds of ethyl acetate fraction to the erythrocytes and the reticulocytes is the same. Reticulocytes are young erythrocyte cells and will develop into erythrocytes.

The average content of hemoglobin (g/dl) determined after 14 days implementation of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. It could be seen in Table 9. The statistical analysis result (Table 10) showed that there was a significant effect of doses application as well as a period of monitoring (application length) on the content level of hemoglobin after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). Trough DMRT result (Table 11 and Table 12) it showed that the effect of dose 40 mg/kgBW and 63.2 mg/kgBW was not significantly different with the positive control, while to dose 100 mg/kgBW, was significantly increasing the value of hemoglobin ($p<0.05$). Hemoglobin was a substance that contains iron ion called hem (heme) and globulin protein. There are around 300 hemoglobin in one red blood cells. Hemoglobin functions to distribute oxygen from lungs to all over the body. Hemoglobin

also brings carbon dioxide back to the lungs to be blown out of the body[14]. So the differences the doses of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. to the increase of hemoglobin value was really significant.

Monitoring the value of hematocrit was conducted after applying an ethyl acetate fraction of *Myrmecodia tuberosa* Jack. for 14 days, it showed in Table 13. Statistical analysis (Table 14) indicates that there was a significant effect resulting from applying doses (Table 15) and duration of treatment (Table 16) on hematocrit value after applying ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). 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 really significant.

Erythrocyte contains hemoglobin which enables red blood cells to take oxygen from the lungs and distribute it through all over the network of body organs[14]. Mature red blood cells are taken out from the spinal cord and alive for around 120 days so that later undergo disintegration and die. The dead red blood cells were replaced by new cells which produced by a spinal cord[14]. Red blood cells are derived from hemocytoblast cells. New hemocytoblast will continuously form from cell primordial of a spinal cord. Hemocytoblast would form erythroblast basophilic which begin to synthesize hemoglobin, and then erythroblast turns into a form of erythroblast polychromatophilia, then to nucleus cells are getting decreased while hemoglobin was formed more in amount and cells change into a form of normoblast. After cytoplasm normoblast filled with nucleus hemoglobin, it becomes so tiny that it is discarded. At the same time, reticulum endoplasma were reabsorbed by cells, at this moment, cells are named reticulosis because they still contain a few reticulum endoplasma basophilic which stay with hemoglobin inside the cytoplasm. Cells in the stadium of reticulocyte enter capillary

diapedesis (slip into membrane pores). After reticulum is all reabsorbed, cells will become matured erythrocyte[16].

Based on the explanation above, 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, hence it could be used as an alternative medicine for healing anemia.

CONCLUSION

The conclusion of this research was:

a. The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at dose 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW can increase the forming of blood cells.

b. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., the more blood cells will produce.

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Figure 2. Picture of ant hill tuber *Myrmecodia tuberosa* Jack.

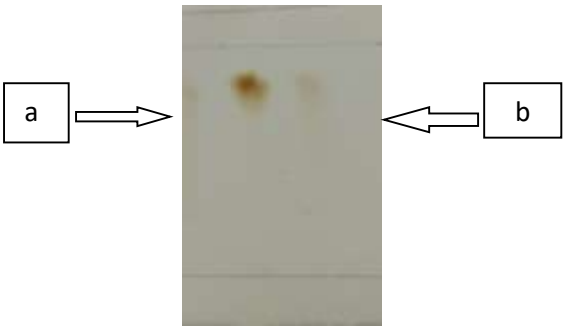


Figure 2. TLC profile of fraction (*Myrmecodia tuberosa* Jack.) below UV 254 nm with ethyl acetate solvent and an eluen mixture of butanol: acetate acid: water (2:0.5:2.5).
Note a = quercetin b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack

Table 1. The calculation result of the number of erythrocyte cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at a different doses within 14 days.

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. The result of two-way ANOVA analysis from erythrocyte value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis of treatment duration on erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14^{th} day	20	4.4050		
21^{st} day	20		5.3040	
28^{th} day	20			5.6690
Sig.		1.000	1.000	1.000

Table 4. The Result of DMRT analysis from erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05		
		1	2	3
The positive control	15	4.8213		
40mg/kgBW	15		5.0447	
63.2mg/kgBW	15			5.2880
100mg/kgBW	15			5.3500
Sig.		1.000	1.000	.405

Table 5. The calculation result of the number of reticulocytes cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at a different doses within 14 days.

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[a]
Dose 40 mg/KgBW	0,48±0,08	0,76±0,11	0,86±0,09	0,70±0,19[b]
Dose 63.2 mg/KgBW	0,44±0,05	0,78±0,08	0,96±0,11	0,73±0,24[b]
Dose 100 mg/KgBW	0,42±0,08	1, 02±0,15	1,38±0,13	0,94±0,43[c]
Average ± SD	0,44±0,07[a]	0,81±0,17[b]	0,99±0,25[c]	

Tabel 6. The result of two-way ANOVA analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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 7. The Result of DMRT analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05		
		1	2	3
The positive control	15	.6267		
40mg/kgBW	15		.7000	
63.2mg/kgBW	15		.7267	
100mg/kgBW	15			.9400
Sig.		1.000	.445	1.000

Table 8. The Result of DMRT analysis of treatment duration on reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	.4400		
21st day	20		.8100	
28th day	20			.9950
Sig.		1.000	1.000	1.000

Table 9. The result of determining the content level of hemoglobin of mice induced with chloramphenicol within 14 days, it continues with applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. tuber with different doses within 14 days.

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 10. The result of two-way ANOVA analysis from hemoglobin value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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 11. The Result of DMRT analysis from hemoglobin value after ethyl acetate fraction *of Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05	
		1	2
The positive control	15	13,9887	
40mg/kgBW	15	14,2380	
63,2mg/kgBW	15	14,3440	
100mg/kgBW	15		15,7987
Sig.		,407	1,000

Table 12. The Result of DMRT analysis of treatment duration on hemoglobin value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	12,0375		
21st day	20		15,3515	
28th day	20			16,3880
Sig.		1,000	1,000	1,000

Table 13. The result of determining hematocrit value of mice induced with chloramphenicol within 14 days, after applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. with different doses within 14 days.

Doses	Value of hematocrit (%)			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 14. The result of two-way ANOVA analysis from hematocrite value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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			

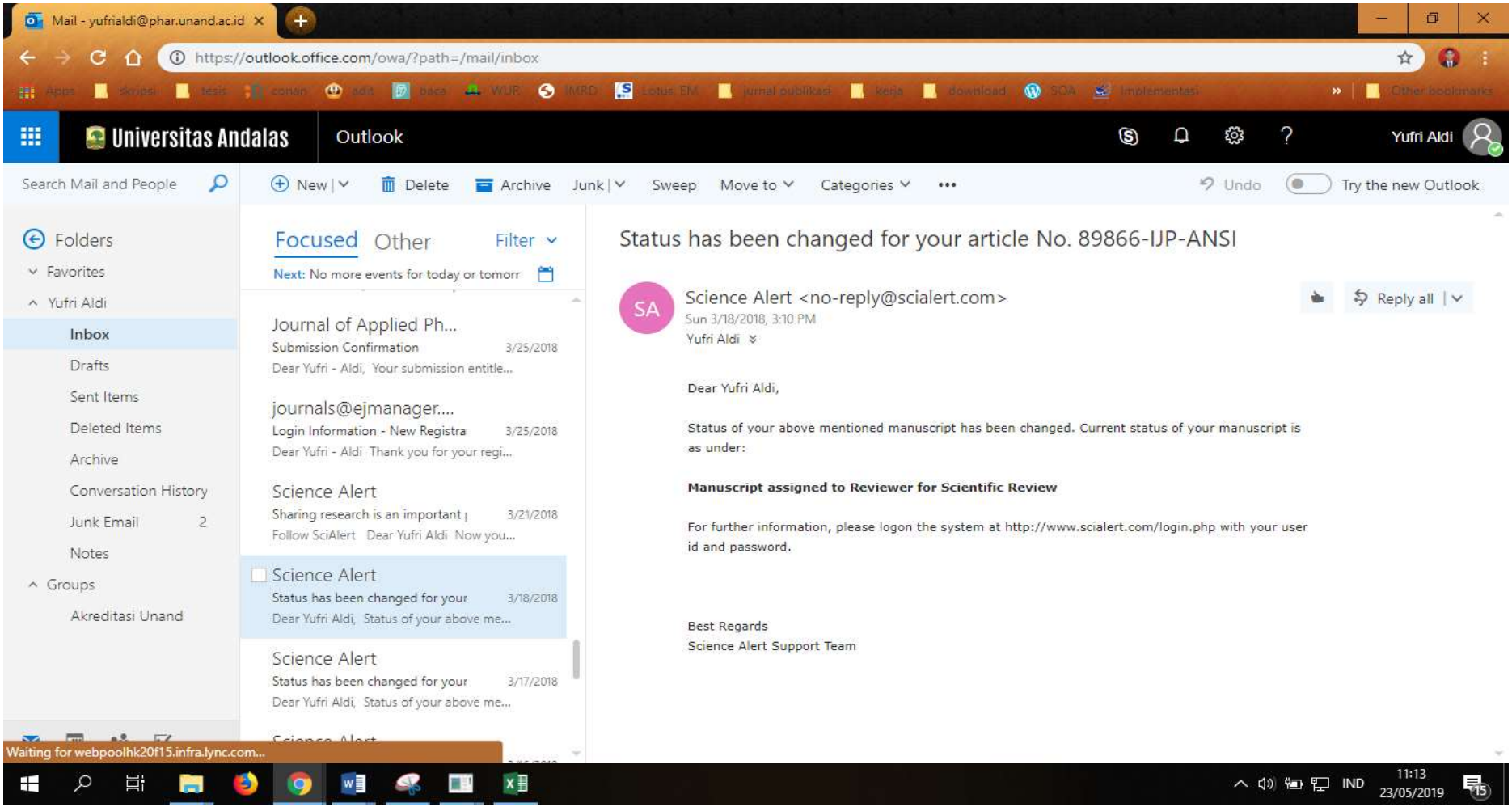
Table 15. The Result of DMRT analysis from hematocrite value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given for 14 days.

Doses	N	Subset for alpha = 0.05	
		1	2
The positive control	15	43,900	
40mg/kgBW	15	45,233	45,233
63,2mg/kgBW	15	45,267	45,267
100mg/kgBW	15		46,467
Sig.		,103	,141

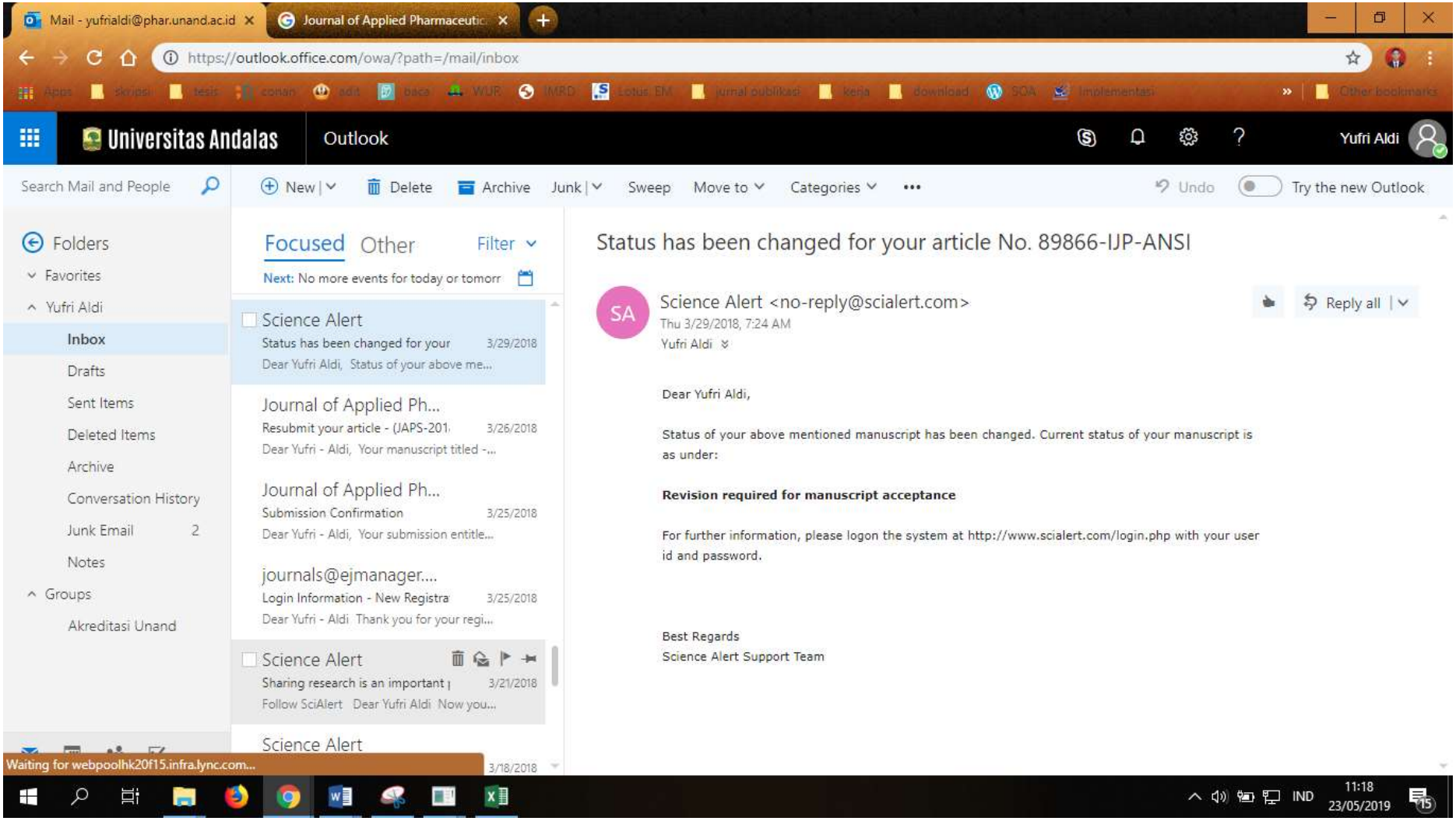
Table 16. The Result of DMRT analysis of treatment duration on hematocrite value after ethyl acetat fraction *Myrmecodia tuberosa* Jack.

Duration	N	Subset for alpha = 0.05		
		1	2	3
14th day	20	43.100		
21st day	20		45.275	
28th day	20			47.275
Sig.		1.000	1.000	1.000

9. Status artikel berubah menjadi **“Manuscript assigned to Reviewer for Scientific Review” (18 Maret 2018).**



10. Status artikel berubah menjadi **"Revision Required for Manuscript Acceptance" –Revisi 3- (29 Maret 2018)**



11. Menyerahkan Revisi 3 pada pihak Jurnal melalui laman portal http://www.scialert.com/

Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack in Anemia
(Effect of treatment of *Myrmecodia tuberosa* Jack. on anemia mice)

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LiveDNA: 62.23458

ABSTRACT

Background and Objective: Ant plant is a Hydropytinae plant (Rubiaceae) that symbiotic with ants and epiphytic inherent in other plants and does not live parasitically in its host. This plant has been used as a blood increaser and commonlyt used to counteract lack of blood during giving birth in the tribe of Mentawai Island. The result of previous research reported that the activities ethyl acetate fraction of *Myrmecodia tuberosa* Jack could improve the to increase phagocytes activity from macrophage and lymphocyte proliferation, also could prevent anaphylactic reaction of active cutaneous. Thus, based on that circumstance, the objective of this research was to continue the previous research to determine the effect of acetate fraction *Myrmecodia tuberosa* Jack. on blood cell formation by counting the amount of erythrocyte, reticulocyte, hemoglobin content as a parameter and the value of hematocrit on the mice. **Materials and Methods:** The research was conducted in 3 months and consists of a group control positive and 3 groups of treatment of an ethyl acetate fraction with 3 various doses. The mice were inducted with chloramphenicol 130 mg/kgBW for first 14 days in order to make them suffered anemia then for next 14 days it gave a fraction of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. orally with variation doses: 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW. Monitoring of mice condition was conducted on day 0, 14, 21 and 28. The

data were analysed with two-direction ANOVA then analysed with DMRT at a significance level of $p<0.05$ **Results:** The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. significantly increased the erythrocyte, reticulocyte, hemoglobin, hematocrit at 14^{th} days with doses 40 mg/kgBW–63.2 mg/kgBW ($p<0.01$). **Conclusions:** The research showed that 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, hence it could be used as an alternative medicine for healing anemia.

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

INTRODUCTION

Indonesia is in the second place after Brazil which has the highest biodiversity of the plant. Among of that, 1,845 plants were known as nitrous medicines that have been used as traditional medicine by many ethnics especially the society in villages on Indonesia[1,2].

One of the medicinal plants that very efficacious for the health was ant nest plant or usually called *Myrmecodia sp*. Commonly the tuber and nest plants that were used as the part of medication are *Myrmecodia tuberosa*, *Myrmecodia pendants* and *Hydnophytum formicarum* (*Rubiaceae*)[3]. It consists of many chemical compounds such as flavonoids, triterpenoid, tocopherol, polyphenol, glycoside, tannin and also various minerals such as calcium, sodium, calcium, zinc, iron, phosphorus and magnesium[4,5].

The previous research showed the potential contents of ethyl acetate fraction of *Myrmecodia tuberosa* could increase phagocytes activity of macrophages and lymphocyte proliferation in vitro[6], it was related to phenol and flavonoids group compound that could increasing lymphocyte cell proliferation then became anti-cancer agent[7] and could prevent the reaction of active Cutaneous nephrotoxic[8]. Ethanol extract of *Myrmecodia tuberosa in vivo* could increase TCD4+ and TCD8+ in mice SD (Sprague Dawley) after they given doxorubicin[9]. Flavonoid of *Myrmecodia tuberosa* could decrease an inflammation with the most optimal anti-inflammatory power[10]. Terpenoid compound of *Myrmecodia tuberosa* was used as anti-cancer especially cervical cancer[11]. N-hexane fraction and water of *Myrmecodia tuberosa* ethanol extract showed significant percentage death of tongue cancer SP-C1[12]. Also, extract water of *Myrmecodia tuberosa* could healing diarrhea and increase feces consistency[13].

The Red blood cells mature is taken out by bone marrow and live about 120 days to have disintegration then die. Dead red blood cells are replaced by new cells which are produced by bone marrow. The White blood cells are different from erythrocytes because there are

nucleus and have independent movement. It made by bone marrow and lymph nodes to eradicate germs disease[14].

Blood depiction is one of concern in health status because blood is component that has important functions in maintaining the physiological body. In general, the function of blood was related to transport component in the body such as nutrient, oxygen, carbon dioxide, metabolites, hormone, head and immune body. Whereas, other function of blood-related to fluid balance and body pH[15].

Anemia was the condition lack of blood or red blood cell ability to carry oxygen that happened because of decreasing number of red blood cells. Anemia also happens when the hemoglobin level is under 12 g/dl in a woman and 14 g/dl in a man, diagnosis of anemia was not only observed by the numbers of erythrocytes and hemoglobin level but also hematocrit value and numbers of reticulocytes. Calculating hematocrit is important to find out types of anemia and it is used to find out spinal cord activities, where is the production place of red blood cells[16,17].

Based on that explanations, the research about ants' nest activity especially the species of *Myrmecodia tuberosa* Jack. toward red blood cells have not been conducted yet. Thus, this research was aim to determine the influence of ethanol extract fraction in *Myrmecodia tuberosa* Jack. activity toward component red blood cells formation with various observed in hematological parameters which are the number of erythrocytes, reticulocyte, hemoglobin level and hematocrit value that are done to mice that has an anemia.

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.

Tools

The tools that were used animal scales, maceration bottle, mortar, stamper, mice cage, measuring glass, sonde needles, thin-layer chromatography (TLC) plate, hematocrit pipette, hemoglobin pipette, hettich centrifuge, spectrophotometer *Uv-Visible* (BIO-RADx Mark) erythrocytes pipette, hemocytometer and microscope (ZEISS).

Animal experimentation

The twenty of mice (*Mus muculus, Swiss webster strain)* was used which is 2-3 months old and weight about 20-30g (It is available in Pharmacology Laboratory, Faculty of Pharmacy Universitas Andalas). Before used, it was acclimatized for 7 days to observe animal behaviour.

Extraction and fractionation *Myrmecodia tuberosa* Jack.

The four kg of fresh *Myrmecodia tuberosa* Jack., slices into to 2-3 mm, then dried in a greenhouse for 3 days, and drying continued the oven at 50°C for 3 days. Thin slices of the *Myrmecodia tuberosa* Jack. has been dried and then blended, thus obtained 400g of *Myrmecodia tuberosa* Jack. powder. The powder of *Myrmecodia tuberosa* Jack. put it into dark macerator bottle, added 4L of 70% ethanol solvent. Its soaked for three days, stir occasionally and separate macerate by filtering with filter paper, repeat the filtering process for four times until the color becomes clear. Collect all macerate then evaporated *in vacuo* with a rotatory evaporator until obtained a thick extract[18,19].

Thick extract of *Myrmecodia tuberosa* Jack. soluted with aquades and added ethyl acetate solvent with the same volume and insulated the ethyl acetate solvent then evaporated until obtained a congealed fraction of *Myrmecodia tuberosa* Jack.

The Congealed Fraction of Ethyl Acetate Characterization of *Myrmecodia tuberosa* Jack.

The congealed fraction of ethyl acetate was determined by organoleptic test, rendement test, a decrease of drying and ash, and the determination of its TLC profile

The Treatment of Animal Experimentation

To make the anemia mice, 130 mg/ kgBW dose of chloramphenicol was given every day for 14 days. Chloramphenicol gave a pressing spinal in order to hinder proliferation and differentiation so that the forming of red blood cells component was preventable and causing anemia[20.] The anemia mice were divided into four group. The first group was positive control was given physiological NaCl solution then second, third and fourth groups were given a treatment of fraction of ethyl acetate of *Myrmecodia tuberosa Jack*. with each of the dose were 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW.

The Determination of Erythrocyte Number

Erythrocytes pipette at first was rinsed used Hayem solution, the tail of mice was cut off, clean the blood used a cotton and let the bleeding, then suction with pipette until the line of 0.5 µl, the blood that was in a tip of a pipette was cleaned with the tissue. Put the pipette in Hayem solution while holding the blood in line 0.5 µl, then it was sucked Hayem solution until the line of 101 µl. The pipette was lifted from solution, the cover of pipette tip using fingers and rubber suction tools was released. The filled pipette was shaken in 3 minutes, and first and second times drops were thrown away and the pipette tip was touched in surface counting space. Set aside the solution for 2-3 minutes in order erythrocyte settled. The number of erythrocytes was counted under the microscope with 400x enlargement[17,21].

Calculating Reticulocyte

Put the blood and dye (brilliant cresyl blue) into the tube with ratio 1:1, mix it well, set aside for 15 minutes so that the dye becomes perfect. Put the 1-2 drops of solution into blood film, let it dry. Examined it under the microscope with the enlargement of 100x to make sure the solution has contained reticulocyte that showed by blue granules/filament and erythrocyte showed by light blue. Count the number of reticulocytes in 1000 cells erythrocyte [17,21].

Determination of Hemoglobin Level

Put 5 ml Drabkin solution into a tube. Took mice's blood from vein tail 20 μL, then put it in the tube. Shake the tube until 2 solutions were blended then set aside at room temperature for 3 minutes. Determine the absorbance value using spectrophotometer in the wavelength 546 nm[17,21].

Determination of Hematocrit Value

Took mice's blood then put it in pipette microcapillary until filled ¾ and one of a tip of the pipette was covered with a candle. Put microcapillary tube into centrifugation (microhematocrit centrifuge), then centrifuged at 16000 rpm for 5 minutes. Measure the height erythrocytes and the high of whole solution that is in microcapillary pipette.

Data Analysis

To determine the effect of a fraction of ethyl acetate and duration of treatment to erythrocyte value, reticulocyte, hemoglobin, and hematocrit were analysed with two-way ANOVA then continued with Duncan Multiple Range Test (DMRT) with IBM SPSS V20.0 and the result are considered significant at $p<0.05$.

RESULT AND DISCUSSION

Result

The result of organoleptic indicated that the fraction was congealed, aromatic, the color was black-brown, and the taste was bitter. The ethyl acetat fraction obtained 5.59% rendement. The decrease of drying and ash content from ethyl acetate fraction of *Myrmecodia tuberosa* Jack. were 11.44% and 6.24%. To determine TLC profile of ethyl acetate fraction of *Myrmecodia tuberosa* Jack., eluen the mixture of butanol : acetate acid : water (2:0.5:2.5). Signing compound which is used was quercetin. The obtained value of RF was 0.78 and its TLC profile could be seen in Figure 2.

Determining the average amount of erythrocyte (million/µl) was done after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied within 14 days and the result showed in Table 1. The result of statistical analysis (Table 2) suggests that the amount of erythrocyte after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied to all doses groups, the duration of the application and interaction between doses and duration of application was increased significantly ($p<0.01$). The effect resulting from doses 63.2 and 100 mg/kgBW was the same as the increasing amount of erythrocyte ($P<0.01$). The result of DMRT Test (Table 3) showed that the effect of each dose and duration of treatment significantly increase erythrocyte value ($p<0.01$). The bigger doses and the longer treatment of the ethyl acetate fraction given, the more visible the effects that occur, but the doses 63.2 mg/kgBW and 100 mg/kgBW was not significantly different.

The amount of reticulocyte after the application of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. within 14 days shown in Table 4. could increase the amount of reticulocyte significantly ($P<0.01$). The effect that caused by dose 40 mg/kgBW was the same as doses 63.2 mg/kgBW and 100 mg/kgWB was significantly different ($p<0.01$).

The activity doses of ethyl acetate fraction and duration of treatment of reticulocyte could be seen in Table 5 and Table 6. It showed the same effect happened same as the effect on the number of erythrocytes above which mean the work of the compounds of ethyl acetate fraction to the erythrocytes and the reticulocytes is the same. Reticulocytes are young erythrocyte cells and will develop into erythrocytes.

The average content of hemoglobin (g/dl) determined after 14 days implementation of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. It could be seen in Table 7. The statistical analysis result (Table 8) showed that there was a significant effect of doses application as well as a period of monitoring (application length) on the content level of hemoglobin after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). Trough DMRT result (Table 9) it showed that the effect of dose 40 mg/kgBW and 63.2 mg/kgBW was not significantly different with the positive control, while to dose 100 mg/kgBW, was significantly increasing the value of hemoglobin ($p<0.05$).

Monitoring the value of hematocrit was conducted after applying an ethyl acetate fraction of *Myrmecodia tuberosa* Jack. for 14 days, it showed in Table 10. Statistical analysis (Table 11) indicates that there was a significant effect resulting from applying doses and duration of treatment (Table 12) on hematocrit value after applying ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). 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 really significant.

Discussion

Anemia is a condition in which blood or red blood cells ability to carry oxygen was declining, usually caused by decreasing amount of circulating red blood cells. Anemia also occurs whenever the content level of hemoglobin was below 12 g/dl for female and 14 g/dl for

male, it also showed from the value of hematocrit as well as an amount of reticulocyte in a low condition[22]. Calculating the value of hematocrit was necessary to find out types of anemia and calculating reticulocyte was useful to see activities of the spinal cord, in which red blood cell is produced. Erythrocytes are the most common cells compared with the other 2 cells (leukocytes and platelets). Erythrocytes contain hemoglobin which allows red blood cells to carry oxygen from the lungs and deliver it throughout the body tissues[23]. The mature of blood cells are removed from the bone marrow and live about 120 days to disintegrate and die. The dead red blood cells are replaced by new cells produced by the bone marrow[14,24].

To determine the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit was conducted on day 14, 21 and 28. In each group of mice, the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit could be seen after 14 days of giving chloramphenicol, then will increase on day-21 and 28 after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied[25].

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 red blood cell components can be inhibited and cause anemia. Anemia caused by chloramphenicol is classified into aplastic anemia. Anema aplastic is a deficiency of erythrocytes, reticulocytes, hemoglobin and hematocrit inability of spinal cord producing erythroblast cells[20,26].

The increasing of calculation reticulocyte suggest that nothing matters with the function of the spinal cord or erythropoietin stimulus[27]. This has proved that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was able to stimulate spinal in order to produce reticulocyte[28]. An increase in the number of reticulocytes in peripheral blood describes accelerated erythrocyte production in bone marrow. Otherwise, a low reticulocyte count may indicate a state of bone marrow hypofunction or aplastic anemia[29,30].

Hemoglobin was a substance that contains iron ion called hem (heme) and globulin protein. There are around 300 hemoglobin in one red blood cells. Hemoglobin functions to distribute oxygen from lungs to all over the body. Hemoglobin also brings carbon dioxide back to the lungs to be blown out of the body[14]. So the differences the doses of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. to the increase of hemoglobin value was really significant.

Erythrocyte contains hemoglobin which enables red blood cells to take oxygen from the lungs and distribute it through all over the network of body organs[30]. Mature red blood cells are taken out from the spinal cord and alive for around 120 days so that later undergo disintegration and die. The dead red blood cells were replaced by new cells which produced by a spinal cord[14]. Red blood cells are derived from hemocytoblast cells. New hemocytoblast will continuously form from cell primordial of a spinal cord. Hemocytoblast would form erythroblast basophilic which begin to synthesize hemoglobin, and then erythroblast turns into a form of erythroblast polychromatophilia, then to nucleus cells are getting decreased while hemoglobin was formed more in amount and cells change into a form of normoblast. After cytoplasm normoblast filled with nucleus hemoglobin, it becomes so tiny that it is discarded. At the same time, reticulum endoplasma were reabsorbed by cells, at this moment, cells are named reticulosis because they still contain a few reticulum endoplasma basophilic which stay with hemoglobin inside the cytoplasm. Cells in the stadium of reticulocyte enter capillary diapedesis (slip into membrane pores). After reticulum is all reabsorbed, cells will become matured erythrocyte[16].

Based on the explanation above, 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, hence it could be used as an alternative medicine for healing anemia.

CONCLUSION

The conclusion of this research was:

a. The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at dose 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW can increase the forming of blood cells.

b. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., the more blood cells will produce.

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Figure 3. Picture of ant hill tuber *Myrmecodia tuberosa* Jack.

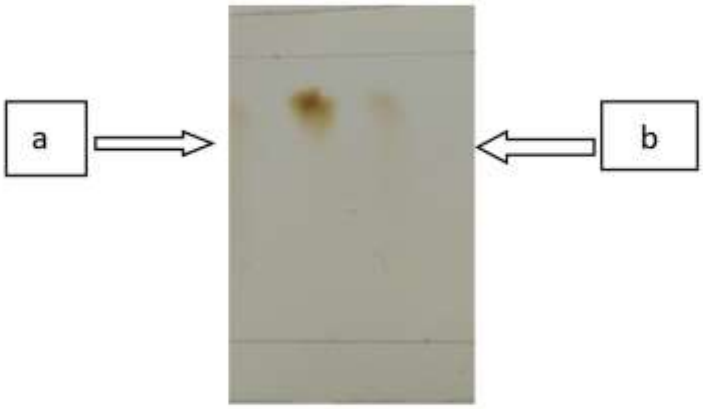


Figure 2. TLC profile of fraction (*Myrmecodia tuberosa* Jack.) below UV 254 nm with ethyl acetate solvent and an eluen mixture of butanol: acetate acid: water (2:0.5:2.5).
Note a = quercetin b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack

Table 1. The calculation result of the number of erythrocyte cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at different doses within 14 days.

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. The result of two-way ANOVA analysis of erythrocyte value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was given.

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. The result of DMRT analysis of erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given

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				
14^{th} day	20	4.4050		
21^{st} day	20		5.3040	
28^{th} day	20			5.6690
Sig.		1.000	1.000	1.000

Table 4. The calculation result of the number of reticulocytes cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at different doses within 14 days.

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. The result of two-way ANOVA analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The result of determining the content level of hemoglobin of mice induced with chloramphenicol within 14 days, it continues with applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. tuber with different doses within 14 days.

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. The result of two-way ANOVA analysis of hemoglobin value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis of hemoglobin value after ethyl acetate fraction *of Myrmecodia tuberosa* Jack. was given.

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				
14^{th} day	20	12.0375		
21^{st} day	20		15.3515	
28^{th} day	20			16.3880
Sig.		1.000	1.000	1.000

Table 10. The result of determining hematocrit value of mice induced with chloramphenicol within 14 days, after applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. with different doses within 14 days.

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	

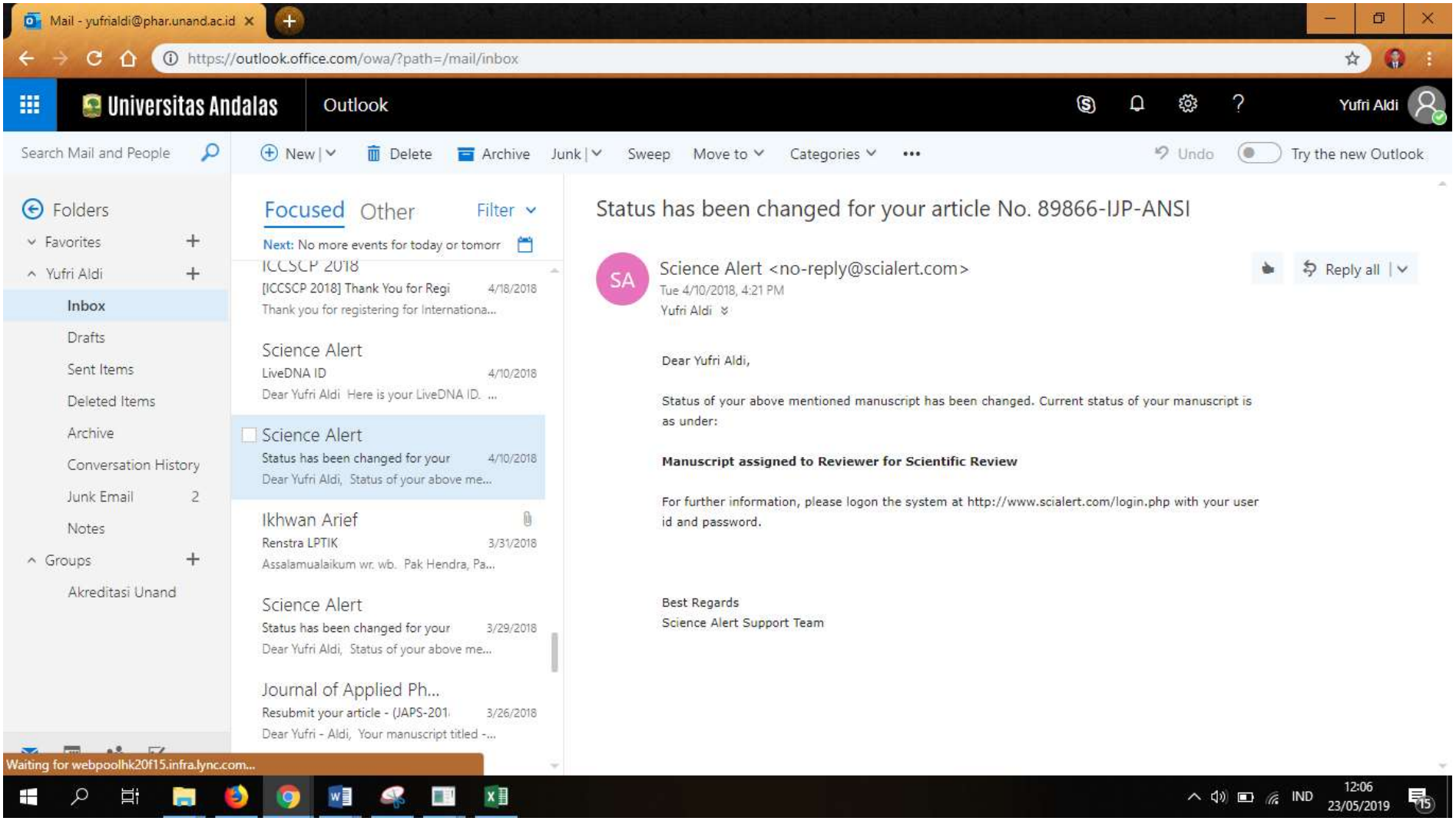
Table 11. The result of two-way ANOVA analysis from hematocrit value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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			

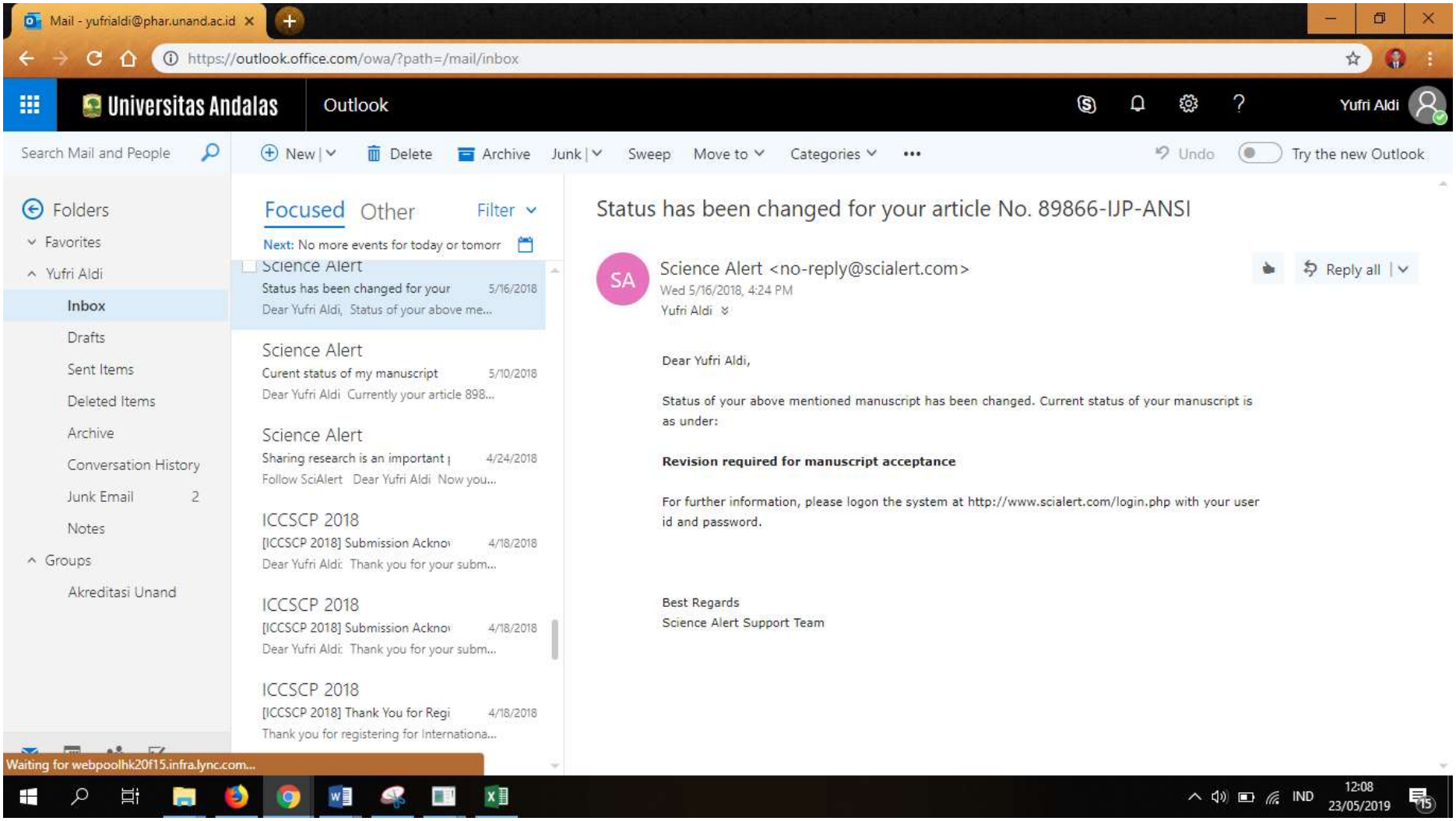
Table 12. The Result of DMRT analysis from hematocrit value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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

12. Status artikel berubah menjadi **“Manuscript assigned to Reviewer for Scientific Review”**



13. Status artikel berubah menjadi “Revision Required for Manuscript Acceptance” –Revisi 4-



14. **Menyerahkan Revisi 4 pada pihak Jurnal melalui laman portal http://www.scialert.com/**

List Modification:

Comment 1: Write of this article is not up to journal standard. We need language editing certificate for this article. **(Last Page)**

Comment 2: Information mentioned under "background and objective" in abstract is unnecessarily prolong do precise this information. **(Line 38-43)**

Comment 3: Provided significance statements is not appropriate. Add a significance statement that must enclosed following information within it;

- What was discovered in this study? **(Line 257-260)**
- What unique point this will provide that benefits scientific community **(Line 261-266)**
- What kind of information this study will provide that could be help in uncovering critical areas that many researcher are not able to explore **(Line 267-274)**
- What kind of new theory will be arrived at? **(Line 267-274)**

Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack in Anemia
(Effect of treatment of *Myrmecodia tuberosa* Jack. on anemia mice)

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ABSTRACT

Background and Objective: The result of previous research reported that the activities ethyl acetate fraction of *Myrmecodia tuberosa* Jack could improve the to increase phagocytes activity from macrophage and lymphocyte proliferation, also could prevent anaphylactic reaction of active cutaneous. Thus, based on that circumstance, the objective of this research was to continue the previous research to determine the effect of acetate fraction *Myrmecodia tuberosa* Jack. on blood cell formation by counting the amount of erythrocyte, reticulocyte, hemoglobin content as a parameter and the value of hematocrit on the mice. **Materials and Methods:** The research was conducted in 3 months and consists of a group control positive and 3 groups of treatment of an ethyl acetate fraction with 3 various doses. The mice were inducted with chloramphenicol 130 mg/kgBW for first 14 days in order to make them suffered anemia then for next 14 days it gave a fraction of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. orally with variation doses: 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW. Monitoring of mice condition was conducted on day 0, 14, 21 and 28. The data were analysed with two-direction ANOVA then analysed with DMRT at a significance level of $p<0.05$ **Results:** The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. significantly increased the erythrocyte, reticulocyte, hemoglobin, hematocrit at 14th days with doses 40 mg/kgBW–63.2 mg/kgBW ($p<0.01$). **Conclusions:** The research showed that 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, hence it could be used as an alternative medicine for healing anemia.

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

INTRODUCTION

Indonesia is in the second place after Brazil which has the highest biodiversity of the plant. Among of that, 1,845 plants were known as nitrous medicines that have been used as traditional medicine by many ethnics especially the society in villages on Indonesia[1,2].

One of the medicinal plants that very efficacious for the health was ant nest plant or usually called *Myrmecodia sp*. Commonly the tuber and nest plants that were used as the part of medication are *Myrmecodia tuberosa*, *Myrmecodia pendants* and *Hydnophytum formicarum* (*Rubiaceae*)[3]. It consists of many chemical compounds such as flavonoids, triterpenoid, tocopherol, polyphenol, glycoside, tannin and also various minerals such as calcium, sodium, calcium, zinc, iron, phosphorus and magnesium[4,5].

The previous research showed the potential contents of ethyl acetate fraction of *Myrmecodia tuberosa* could increase phagocytes activity of macrophages and lymphocyte proliferation in vitro[6], it was related to phenol and flavonoids group compound that could increasing lymphocyte cell proliferation then became anti-cancer agent[7] and could prevent the reaction of active Cutaneous nephrotoxic[8]. Ethanol extract of *Myrmecodia tuberosa in vivo* could increase TCD4+ and TCD8+ in mice SD (Sprague Dawley) after they given doxorubicin[9]. Flavonoid of *Myrmecodia tuberosa* could decrease an inflammation with the most optimal anti-inflammatory power[10]. Terpenoid compound of *Myrmecodia tuberosa* was used as anti-cancer especially cervical cancer[11]. N-hexane fraction and water of *Myrmecodia tuberosa* ethanol extract showed significant percentage death of tongue cancer SP-C1[12]. Also, extract water of *Myrmecodia tuberosa* could healing diarrhea and increase feces consistency[13].

The Red blood cells mature is taken out by bone marrow and live about 120 days to have disintegration then die. Dead red blood cells are replaced by new cells which are produced by bone marrow. The White blood cells are different from erythrocytes because there are nucleus and have independent movement. It made by bone marrow and lymph nodes to eradicate germs disease[14].

Blood depiction is one of concern in health status because blood is component that has important functions in maintaining the physiological body. In general, the function of blood was related to transport component in the body such as nutrient, oxygen, carbon dioxide, metabolites, hormone, head and immune body. Whereas, other function of blood-related to fluid balance and body pH[15].

Anemia was the condition lack of blood or red blood cell ability to carry oxygen that happened because of decreasing number of red blood cells. Anemia also happens when the hemoglobin level is under 12 g/dl in a woman and 14 g/dl in a man, diagnosis of anemia was not only observed by the numbers of erythrocytes and hemoglobin level but also hematocrit value and numbers of reticulocytes. Calculating hematocrit is important to find out types of anemia and it is used to find out spinal cord activities, where is the production place of red blood cells[16,17].

Based on that explanations, the research about ants' nest activity especially the species of *Myrmecodia tuberosa* Jack. toward red blood cells have not been conducted yet. Thus, this research was aim to determine the influence of ethanol extract fraction in *Myrmecodia tuberosa* Jack. activity toward component red blood cells formation with various observed in hematological parameters which are the number of erythrocytes, reticulocyte, hemoglobin level and hematocrit value that are done to mice that has an anemia.

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.

Tools

The tools that were used animal scales, maceration bottle, mortar, stamper, mice cage, measuring glass, sonde needles, thin-layer chromatography (TLC) plate, hematocrit pipette, hemoglobin pipette, hettich centrifuge, spectrophotometer *Uv-Visible* (BIO-RADx Mark) erythrocytes pipette, hemocytometer and microscope (ZEISS).

Animal experimentation

The twenty of mice (*Mus muculus, Swiss webster strain)* was used which is 2-3 months old and weight about 20-30g (It is available in Pharmacology Laboratory, Faculty of Pharmacy Universitas Andalas). Before used, it was acclimatized for 7 days to observe animal behaviour.

Extraction and fractionation *Myrmecodia tuberosa* Jack.

The four kg of fresh *Myrmecodia tuberosa* Jack., slices into to 2-3 mm, then dried in a greenhouse for 3 days, and drying continued the oven at 50°C for 3 days. Thin slices of the *Myrmecodia tuberosa* Jack. has been dried and then blended, thus obtained 400g of *Myrmecodia tuberosa* Jack. powder. The powder of *Myrmecodia tuberosa* Jack. put it into dark macerator bottle, added 4L of 70% ethanol solvent. Its soaked for three days, stir occasionally and separate macerate by filtering with filter paper, repeat the filtering process for four times until the color becomes clear. Collect all macerate then evaporated *in vacuo* with a rotatory evaporator until obtained a thick extract[18,19].

Thick extract of *Myrmecodia tuberosa* Jack. soluted with aquades and added ethyl acetate solvent with the same volume and insulated the ethyl acetate solvent then evaporated until obtained a congealed fraction of *Myrmecodia tuberosa* Jack.

The Congealed Fraction of Ethyl Acetate Characterization of *Myrmecodia tuberosa* Jack.

The congealed fraction of ethyl acetate was determined by organoleptic test, rendement test, a decrease of drying and ash, and the determination of its TLC profile

The Treatment of Animal Experimentation

To make the anemia mice, 130 mg/ kgBW dose of chloramphenicol was given every day for 14 days. Chloramphenicol gave a pressing spinal in order to hinder proliferation and differentiation so that the forming of red blood cells component was preventable and causing anemia[20.] The anemia mice were divided into four group. The first group was positive control was given physiological NaCl solution then second, third and fourth groups were given a treatment of fraction of ethyl acetate of *Myrmecodia tuberosa Jack*. with each of the dose were 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW.

The Determination of Erythrocyte Number

Erythrocytes pipette at first was rinsed used Hayem solution, the tail of mice was cut off, clean the blood used a cotton and let the bleeding, then suction with pipette until the line of 0.5 µl, the blood that was in a tip of a pipette was cleaned with the tissue. Put the pipette in Hayem solution while holding the blood in line 0.5 µl, then it was sucked Hayem solution until the line of 101 µl. The pipette was lifted from solution, the cover of pipette tip using fingers and rubber suction tools was released. The filled pipette was shaken in 3 minutes, and first and second times drops were thrown away and the pipette tip was touched in surface counting space. Set aside the solution for 2-3 minutes in order erythrocyte settled. The number of erythrocytes was counted under the microscope with 400x enlargement[17,21].

Calculating Reticulocyte

Put the blood and dye (brilliant cresyl blue) into the tube with ratio 1:1, mix it well, set aside for 15 minutes so that the dye becomes perfect. Put the 1-2 drops of solution into blood film, let it dry. Examined it under the microscope with the enlargement of 100x to make sure the solution has contained reticulocyte that showed by blue granules/filament and erythrocyte showed by light blue. Count the number of reticulocytes in 1000 cells erythrocyte [17,21].

Determination of Hemoglobin Level

Put 5 ml Drabkin solution into a tube. Took mice's blood from vein tail 20 μL, then put it in the tube. Shake the tube until 2 solutions were blended then set aside at room temperature for 3 minutes. Determine the absorbance value using spectrophotometer in the wavelength 546 nm[17,21].

Determination of Hematocrit Value

Took mice's blood then put it in pipette microcapillary until filled ¾ and one of a tip of the pipette was covered with a candle. Put microcapillary tube into centrifugation (microhematocrit centrifuge), then centrifuged at 16000 rpm for 5 minutes. Measure the height erythrocytes and the high of whole solution that is in microcapillary pipette.

Data Analysis

To determine the effect of a fraction of ethyl acetate and duration of treatment to erythrocyte value, reticulocyte, hemoglobin, and hematocrit were analysed with two-way ANOVA then continued with Duncan Multiple Range Test (DMRT) with IBM SPSS V20.0 and the result are considered significant at $p<0.05$.

RESULT AND DISCUSSION

Result

The result of organoleptic indicated that the fraction was congealed, aromatic, the color was black-brown, and the taste was bitter. The ethyl acetat fraction obtained 5.59% rendement. The decrease of drying and ash content from ethyl acetate fraction of *Myrmecodia tuberosa* Jack. were 11.44% and 6.24%. To determine TLC profile of ethyl acetate fraction of *Myrmecodia tuberosa* Jack., eluen the mixture of butanol : acetate acid : water (2:0.5:2.5). Signing compound which is used was quercetin. The obtained value of RF was 0.78 and its TLC profile could be seen in Figure 2.

Determining the average amount of erythrocyte (million/µl) was done after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied within 14 days and the result showed in Table 1. The result of statistical analysis (Table 2) suggests that the amount of erythrocyte after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied to all doses groups, the duration of the application and interaction between doses and duration of application was increased significantly ($p<0.01$). The effect resulting from doses 63.2 and 100 mg/kgBW was the same as the increasing amount of erythrocyte ($P<0.01$). The result of DMRT Test (Table 3) showed that the effect of each dose and duration of treatment significantly increase erythrocyte value ($p<0.01$). The bigger doses and the longer treatment of the ethyl acetate fraction given, the more visible the effects that occur, but the doses 63.2 mg/kgBW and 100 mg/kgBW was not significantly different.

The amount of reticulocyte after the application of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. within 14 days shown in Table 4. could increase the amount of reticulocyte significantly ($P<0.01$). The effect that caused by dose 40 mg/kgBW was the same as doses 63.2 mg/kgBW and 100 mg/kgWB was significantly different ($p<0.01$).

The activity doses of ethyl acetate fraction and duration of treatment of reticulocyte could be seen in Table 5 and Table 6. It showed the same effect happened same as the effect

on the number of erythrocytes above which mean the work of the compounds of ethyl acetate fraction to the erythrocytes and the reticulocytes is the same. Reticulocytes are young erythrocyte cells and will develop into erythrocytes.

The average content of hemoglobin (g/dl) determined after 14 days implementation of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. It could be seen in Table 7. The statistical analysis result (Table 8) showed that there was a significant effect of doses application as well as a period of monitoring (application length) on the content level of hemoglobin after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). Trough DMRT result (Table 9) it showed that the effect of dose 40 mg/kgBW and 63.2 mg/kgBW was not significantly different with the positive control, while to dose 100 mg/kgBW, was significantly increasing the value of hemoglobin ($p<0.05$).

Monitoring the value of hematocrit was conducted after applying an ethyl acetate fraction of *Myrmecodia tuberosa* Jack. for 14 days, it showed in Table 10. Statistical analysis (Table 11) indicates that there was a significant effect resulting from applying doses and duration of treatment (Table 12) on hematocrit value after applying ethyl acetate fraction of *Myrmecodia tuberosa* Jack. ($p<0.05$). 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 really significant.

Discussion

Anemia is a condition in which blood or red blood cells ability to carry oxygen was declining, usually caused by decreasing amount of circulating red blood cells. Anemia also occurs whenever the content level of hemoglobin was below 12 g/dl for female and 14 g/dl for male, it also showed from the value of hematocrit as well as an amount of reticulocyte in a low condition[22]. Calculating the value of hematocrit was necessary to find out types of anemia and calculating reticulocyte was useful to see activities of the spinal cord, in which red blood cell

is produced. Erythrocytes are the most common cells compared with the other 2 cells (leukocytes and platelets). Erythrocytes contain hemoglobin which allows red blood cells to carry oxygen from the lungs and deliver it throughout the body tissues[23]. The mature of blood cells are removed from the bone marrow and live about 120 days to disintegrate and die. The dead red blood cells are replaced by new cells produced by the bone marrow[14,24].

To determine the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit was conducted on day 14, 21 and 28. In each group of mice, the amount of erythrocyte, reticulocyte, hemoglobin content level and value of hematocrit could be seen after 14 days of giving chloramphenicol, then will increase on day-21 and 28 after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was applied[25].

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 red blood cell components can be inhibited and cause anemia. Anemia caused by chloramphenicol is classified into aplastic anemia. Anema aplastic is a deficiency of erythrocytes, reticulocytes, hemoglobin and hematocrit inability of spinal cord producing erythroblast cells[20,26].

The increasing of calculation reticulocyte suggest that nothing matters with the function of the spinal cord or erythropoietin stimulus[27]. This has proved that ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was able to stimulate spinal in order to produce reticulocyte[28]. An increase in the number of reticulocytes in peripheral blood describes accelerated erythrocyte production in bone marrow. Otherwise, a low reticulocyte count may indicate a state of bone marrow hypofunction or aplastic anemia[29,30].

Hemoglobin was a substance that contains iron ion called hem (heme) and globulin protein. There are around 300 hemoglobin in one red blood cells. Hemoglobin functions to distribute oxygen from lungs to all over the body. Hemoglobin also brings carbon dioxide back

to the lungs to be blown out of the body[14]. So the differences the doses of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. to the increase of hemoglobin value was really significant.

Erythrocyte contains hemoglobin which enables red blood cells to take oxygen from the lungs and distribute it through all over the network of body organs[30]. Mature red blood cells are taken out from the spinal cord and alive for around 120 days so that later undergo disintegration and die. The dead red blood cells were replaced by new cells which produced by a spinal cord[14]. Red blood cells are derived from hemocytoblast cells. New hemocytoblast will continuously form from cell primordial of a spinal cord. Hemocytoblast would form erythroblast basophilic which begin to synthesize hemoglobin, and then erythroblast turns into a form of erythroblast polychromatophilia, then to nucleus cells are getting decreased while hemoglobin was formed more in amount and cells change into a form of normoblast. After cytoplasm normoblast filled with nucleus hemoglobin, it becomes so tiny that it is discarded. At the same time, reticulum endoplasma were reabsorbed by cells, at this moment, cells are named reticulosis because they still contain a few reticulum endoplasma basophilic which stay with hemoglobin inside the cytoplasm. Cells in the stadium of reticulocyte enter capillary diapedesis (slip into membrane pores). After reticulum is all reabsorbed, cells will become matured erythrocyte[16].

Based on the explanation above, 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, hence it could be used as an alternative medicine for healing anemia.

Myrmecodia tuberosa Jack. growing a lot in Mentawai Island and economically have not been using as medicine yet. In this research, we provide the new information about *Myrmecodia tuberosa* Jack. as anemia medicine that cheapest and available a lot in the market.

Furthermore, this research can be directed to determine the activity of active compounds that contained in ethyl acetate fraction of *Myrmecodia tuberosa* Jack. molecularly such as

observation activity of cells under hypoxia condition to cytockine production as erythropoietin (EPO), interleukin-1 (IL-1) and interleukin-9 (IL-9). This cytokine compoud is 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 work specifically against the 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 was:

a. The treatment of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at dose 40 mg/kgBW, 63.2 mg/kgBW and 100 mg/kgBW can increase the forming of blood cells.

b. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., the more blood cells will produce.

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Figure 4. Picture of ant hill tuber *Myrmecodia tuberosa* Jack.

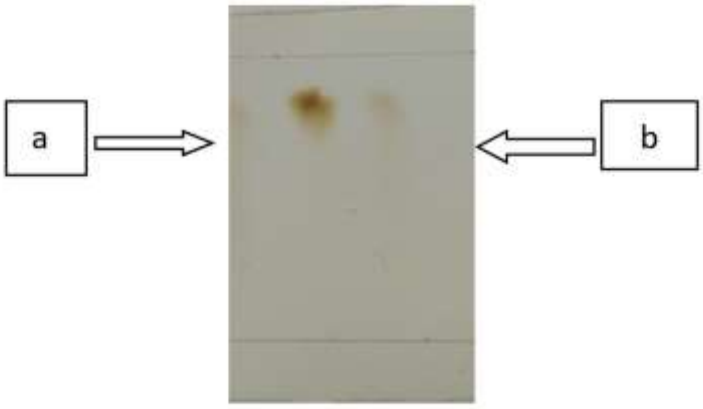


Figure 2. TLC profile of fraction (*Myrmecodia tuberosa* Jack.) below UV 254 nm with ethyl acetate solvent and an eluen mixture of butanol: acetate acid: water (2:0.5:2.5).
Note a = quercetin b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack

Table 1. The calculation result of the number of erythrocyte cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at different doses within 14 days.

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. The result of two-way ANOVA analysis of erythrocyte value after ethyl acetate fraction of *Myrmecodia tuberosa* Jack. was given.

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. The result of DMRT analysis of erythrocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given

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				
14^{th} day	20	4.4050		
21^{st} day	20		5.3040	
28^{th} day	20			5.6690
Sig.		1.000	1.000	1.000

Table 4. The calculation result of the number of reticulocytes cells of the induced in mice with chloramphenicol within 14 days, and continued by given ethyl acetate fraction of *Myrmecodia tuberosa* Jack. at different doses within 14 days.

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. The result of two-way ANOVA analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis from reticulocyte value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The result of determining the content level of hemoglobin of mice induced with chloramphenicol within 14 days, it continues with applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. tuber with different doses within 14 days.

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. The result of two-way ANOVA analysis of hemoglobin value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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. The Result of DMRT analysis of hemoglobin value after ethyl acetate fraction *of Myrmecodia tuberosa* Jack. was given.

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				
14^{th} day	20	12.0375		
21^{st} day	20		15.3515	
28^{th} day	20			16.3880
Sig.		1.000	1.000	1.000

Table 10. The result of determining hematocrit value of mice induced with chloramphenicol within 14 days, after applying suspension of ethyl acetate fraction of *Myrmecodia tuberosa* Jack. with different doses within 14 days.

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	

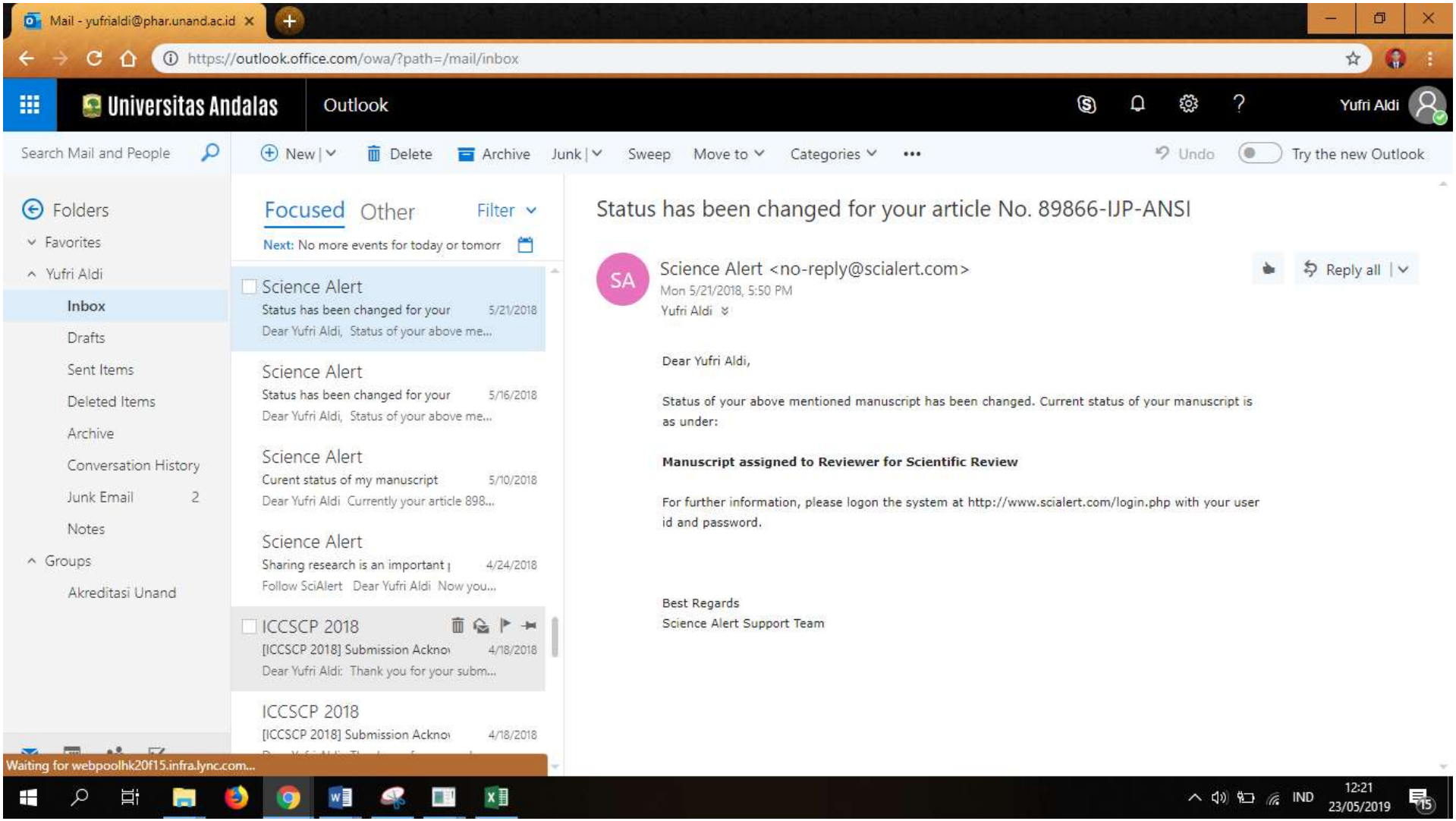
Table 11. The result of two-way ANOVA analysis from hematocrit value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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			

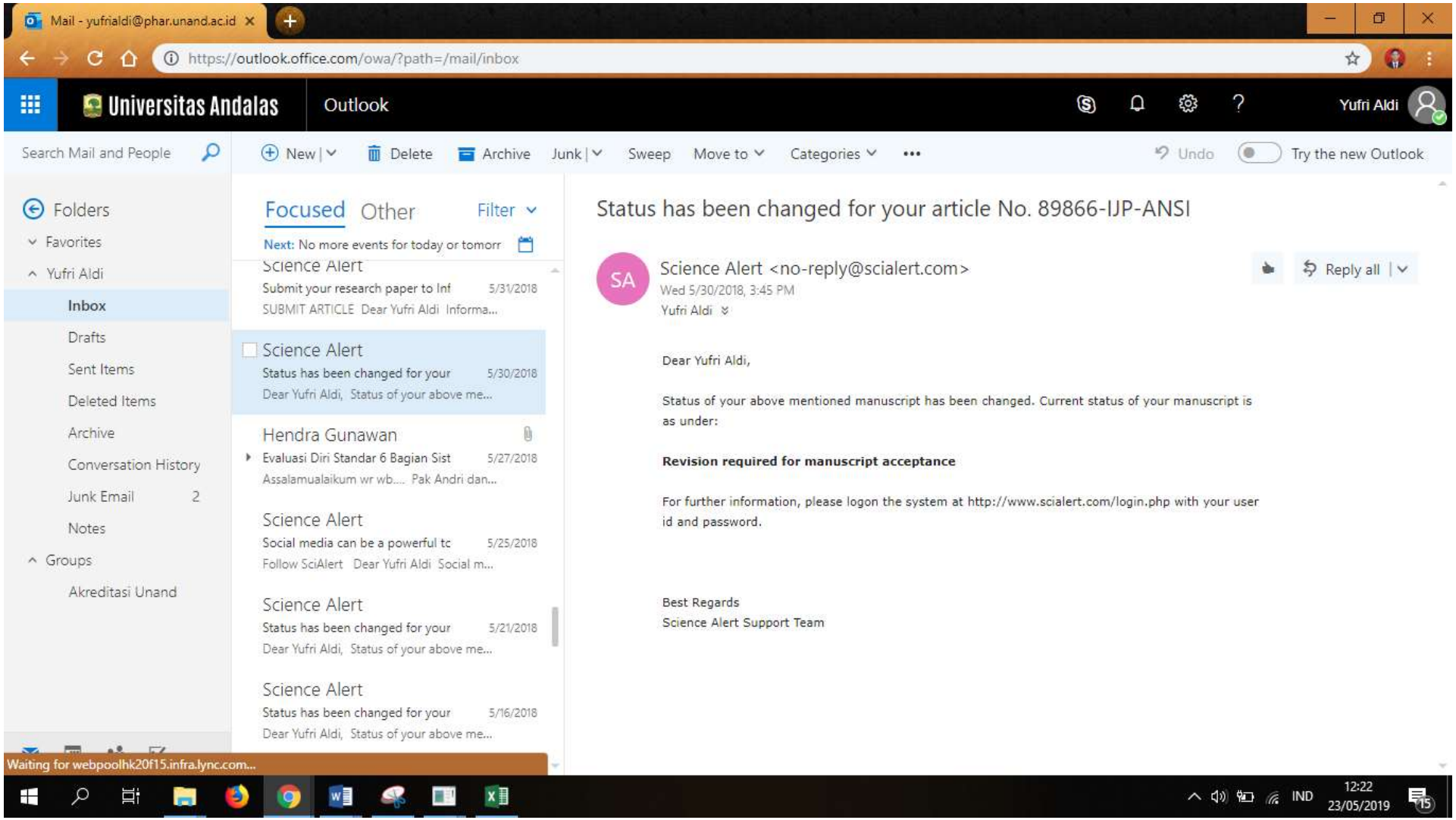
Table 12. The Result of DMRT analysis from hematocrit value after ethyl acetat fraction of *Myrmecodia tuberosa* Jack. was given.

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				
14^{th} day	20	43.100		
21^{st} day	20		45.275	
28^{th} day	20			47.275
Sig.		1.000	1.000	1.000

15. Status artikel berubah menjadi “Manuscript assigned to Reviewer for Scientific Review”



16. Status artikel berubah menjadi “Revision Required for Manuscript Acceptance” –Revisi 5-



17. Menyerahkan **Revisi 5** pada pihak Jurnal melalui laman portal http://www.scialert.com/

/89866-IJP-ANSI / Research Article

Final Decision: Accepted After Minor Revision

Reference your article entitled "Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack. In Anemic Mice" submitted for publication to International Journal of Pharmacology. Before final publication I will suggest you to incorporate the following suggested modifications in your paper and resubmit for further evaluation. My decision is based on the following reason(s):

MAJOR comments in support of the decision

Comment 1: I did some modification at the end of main title and short title because the previous sentence is not suitable. Please check it.

Comment 2: Precisely concise the background of the study under the heading of the Abstract. Summarize it within 1-2lines only.

Comment 3: Author is suggested to discuss the results of current research in the beginning of Discussion section and then co-relate with recent published literature.

Comment 4: Author is suggested to provide the novelty assertion of your current Research work in the form of a statement in the start of the article. Author can also provide at the end under a specific and separate heading of Significance Statement. The significance statement should provide the novel aspect and significance of this research work with respect to the existing literature and more generally to the society. It should be a short summary which describe what this paper adds to and what was already known.

Start this statement with the following words: This study discover the ---------- that can be beneficial for …… **And the last sentence of this statement could be such as**: This study will help the researcher to uncover the critical areas of ---------- that many researchers were not able to explore. Thus a new theory on ---------- may be arrived at.

Author Guidance: Author is advised to do all above mentioned modification in their Corresponding sections in the manuscript, not in the reply of Reviewer's Comments and also Highlighted that modified portion of the manuscript.

NOTE:- Author is guided to do modifications only in this attached manuscript and submit it. Donot attached a new revised copy of the article.

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.

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[18,19].

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[20]. 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[17,21].

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 [17,21].

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 [17,21].

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 [14,24]. Erythrocytes contain hemoglobin which allows red blood cells to carry oxygen from the lungs and deliver it throughout the body tissues[23]. 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 classified as aplastic

anemia. Anema aplastic is a deficiency of erythrocytes, reticulocytes, hemoglobin, and hematocrit as a result of reduction of erythroblast cells being produced in the bone marrow[20,26].

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

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

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

The increase in reticulocyte count suggests that, as expected, chloramphenicol only caused reversible suppression of the bone marrow function and did not permanently damage its ability to produce erythropoietin[27]. On the contrary, the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. appears to stimulate reticulocyte production in the bone 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[29,30].

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.

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.

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

a. 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.
b. The higher the doses of ethyl acetate fraction *Myrmecodia tuberosa* Jack., faster erythrocytes are produced.
c. This suggests that *Myrmecodia tuberosa* Jack. has potential as an economic and effective source of treatment for some types of anemia.

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Figure 5. A fresh “ant nest” tuber *Myrmecodia tuberosa* Jack.

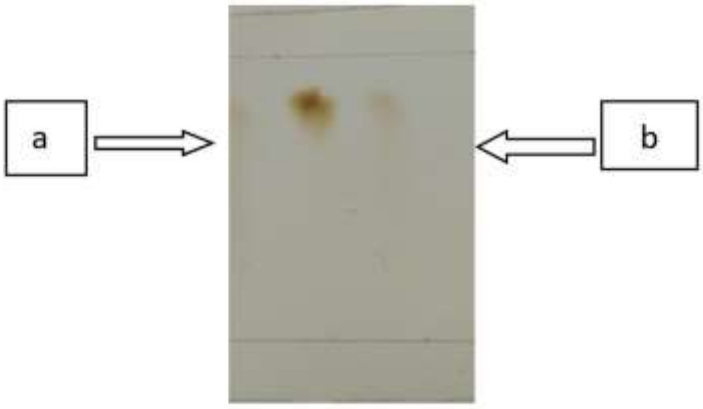


Figure 2. TLC profile of the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. under UV light (254 nm) using an eluent mixture of butanol: acetate acid: water (2:0.5:2.5). Note a = quercetin standard b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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	

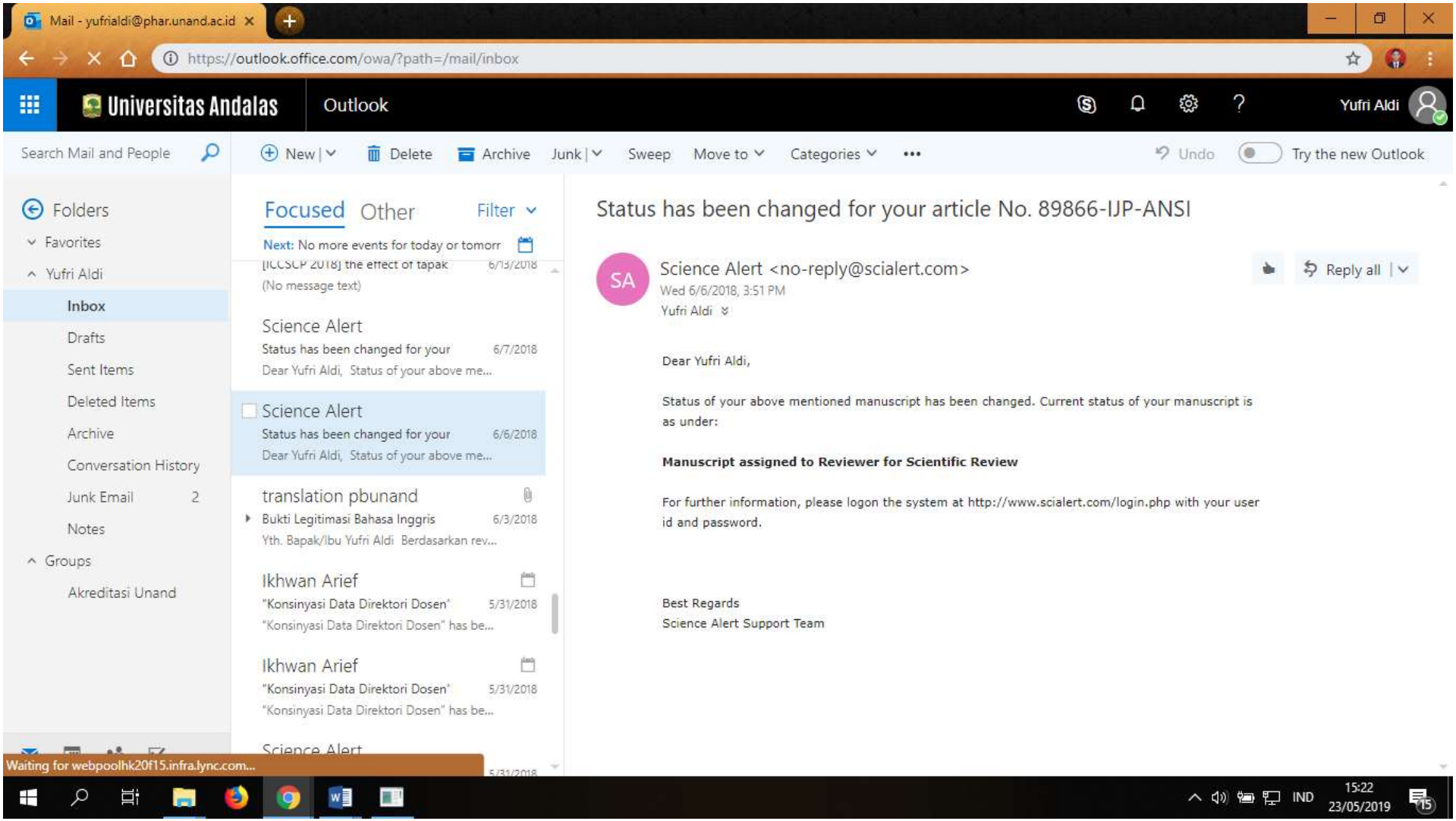
Table 11. Two-way ANOVA analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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			

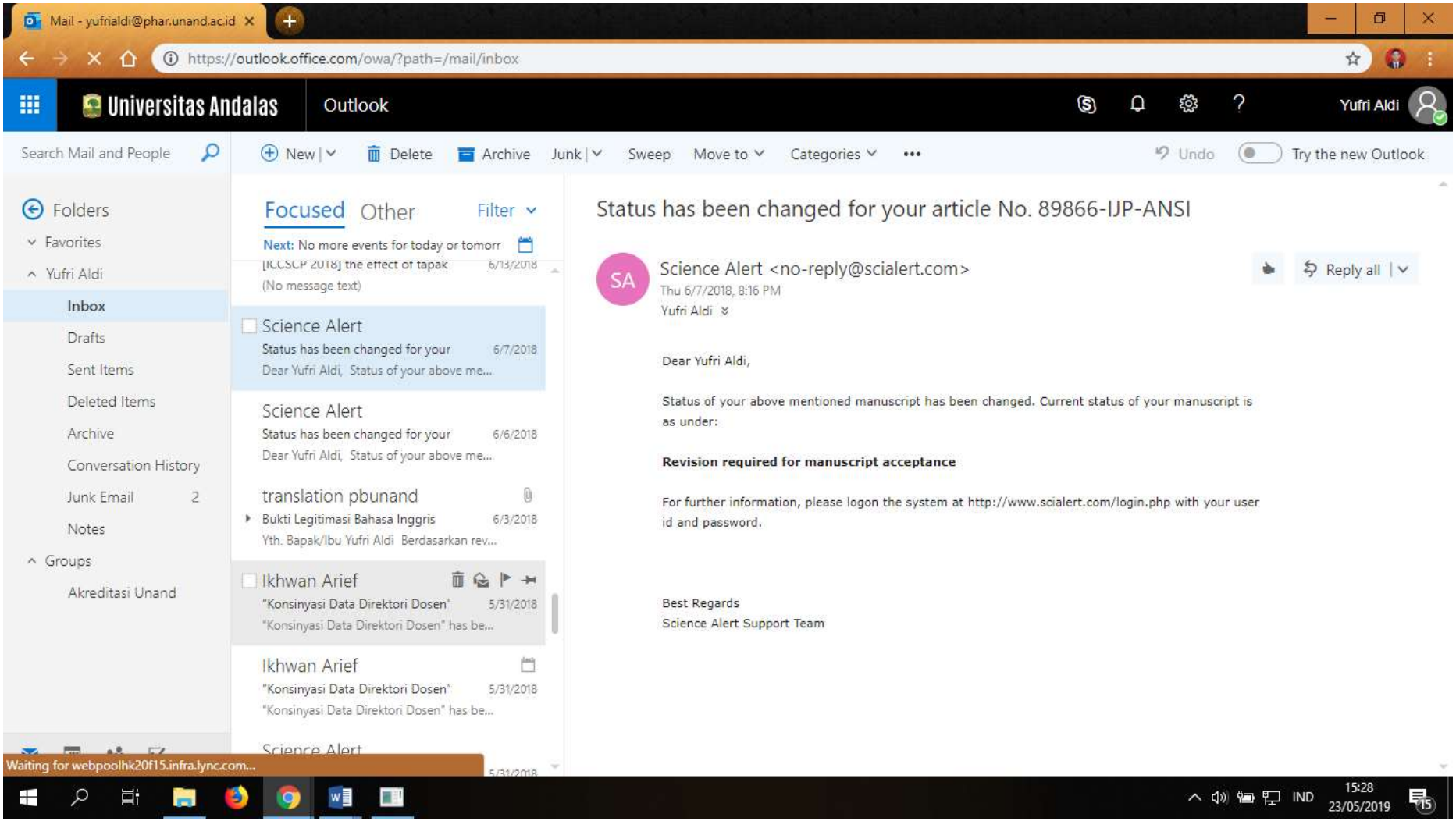
Table 12. DMRT analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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

18. Status artikel berubah menjadi “Manuscript assigned to Reviewer for Scientific Review”



19. Status artikel berubah menjadi “Revision Required for Manuscript Acceptance” –Revisi 6-



20. **Menyerahkan Revisi 6 pada pihak Jurnal melalui laman portal http://www.scialert.com/**

89866-IJP-ANSI / Research Article

Final Decision: Accepted After Minor Revision

Reference your article entitled "Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack. In Anemic Mice" submitted for publication to International Journal of Pharmacology. Before final publication I will suggest you to incorporate the following suggested modifications in your paper and resubmit for further evaluation. My decision is based on the following reason(s):

Minor comments in support of the decision

Comment 1: Quote reference for "Anemia occurs frequently because of malnutrition leading to deficiency in iron, folic acid, or B12 but it can also be a result of damage to the stomach or compromised renal function leading to reduced erythropoietin production and infection.

Comment 2: If possible combine Tables having similar parameters in columns and rows in order to minimize total number of tables and to presented large data in fewer table.

Comment 3: Provide conclusion in form of a paragraph.

Author Guidance: Author is advised to do all above mentioned modification in their Corresponding sections in the manuscript, not in the reply of Reviewer's Comments and also Highlighted that modified portion of the manuscript.

NOTE:- Author is guided to do modifications only in this attached manuscript and submit it. Donot attached a new revised copy of the article.

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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LiveDNA: 62.23458

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 14^{th} 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 classified as aplastic

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

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

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

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

The increase in reticulocyte count suggests that, as expected, chloramphenicol only caused reversible suppression of the bone marrow function and did not permanently damage its ability to produce erythropoietin[29]. On the contrary, the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. appears to stimulate reticulocyte production in the bone 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 6. A fresh “ant nest” tuber *Myrmecodia tuberosa* Jack.

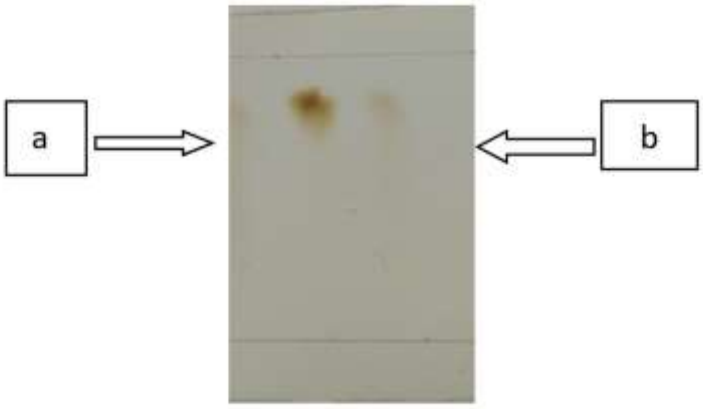


Figure 2. TLC profile of the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. under UV light (254 nm) using an eluent mixture of butanol: acetate acid: water (2:0.5:2.5). Note a = quercetin standard b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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	

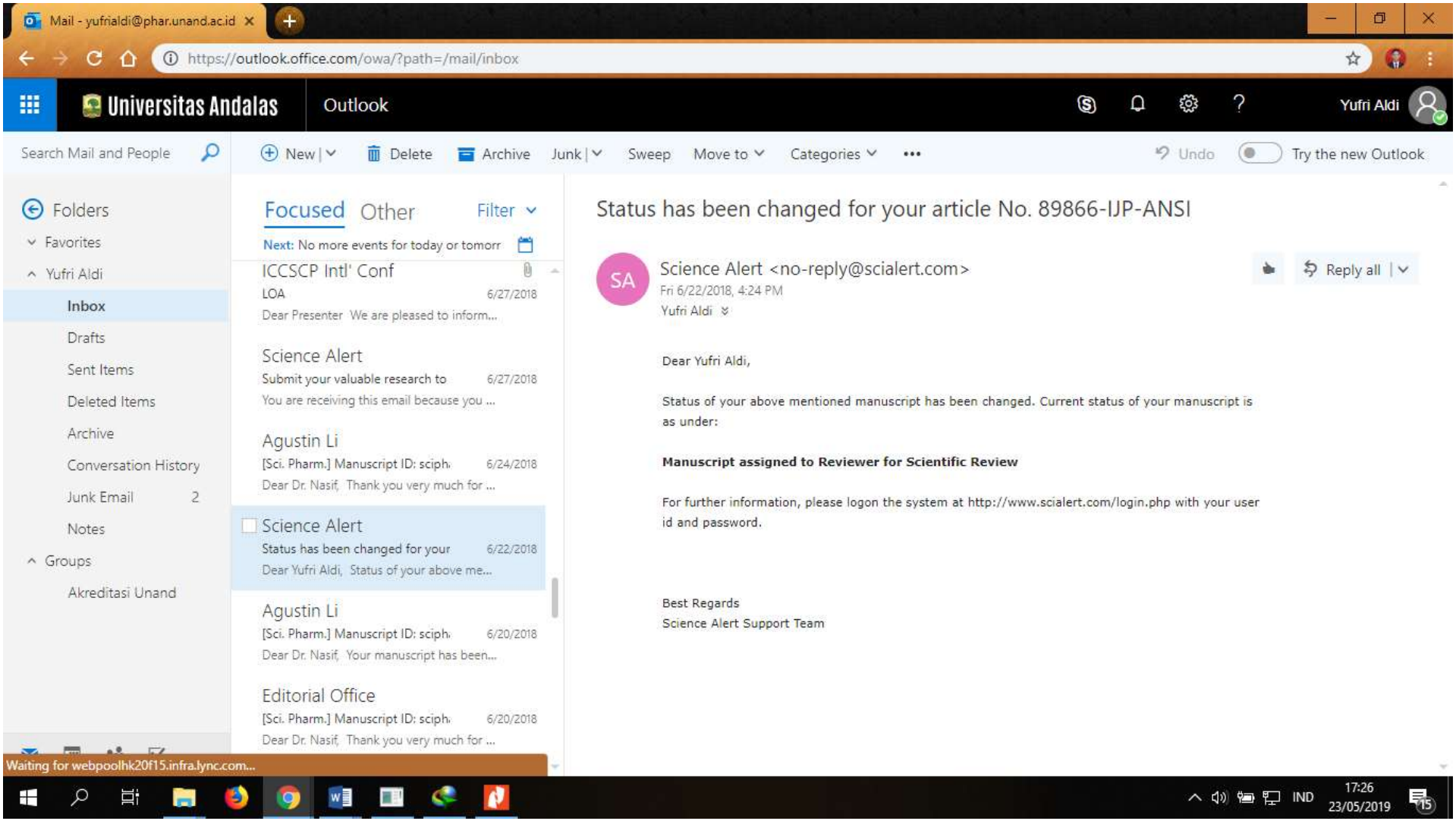
Table 11. Two-way ANOVA analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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			

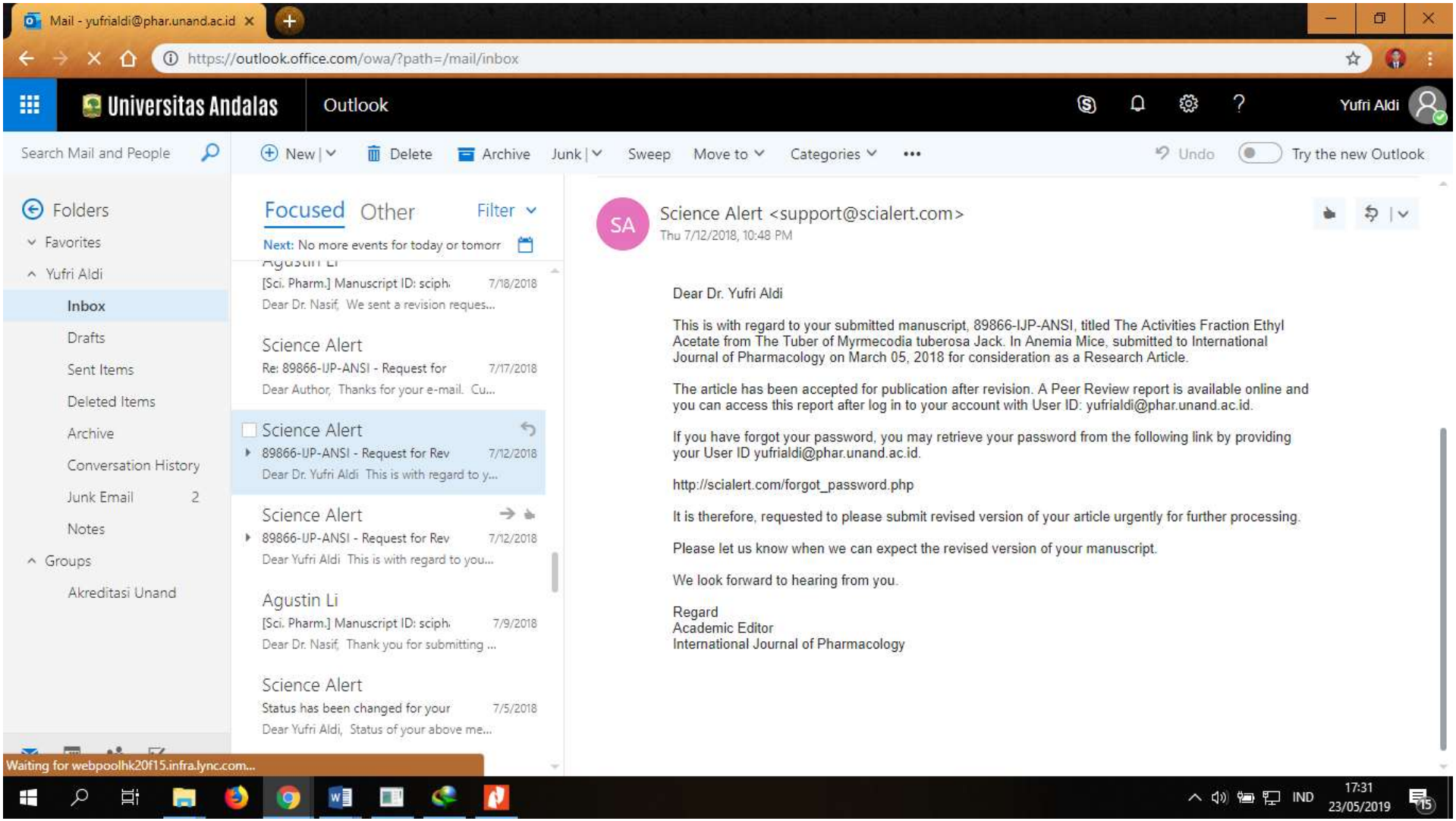
Table 12. DMRT analysis of hematocrit values after dosing with ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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

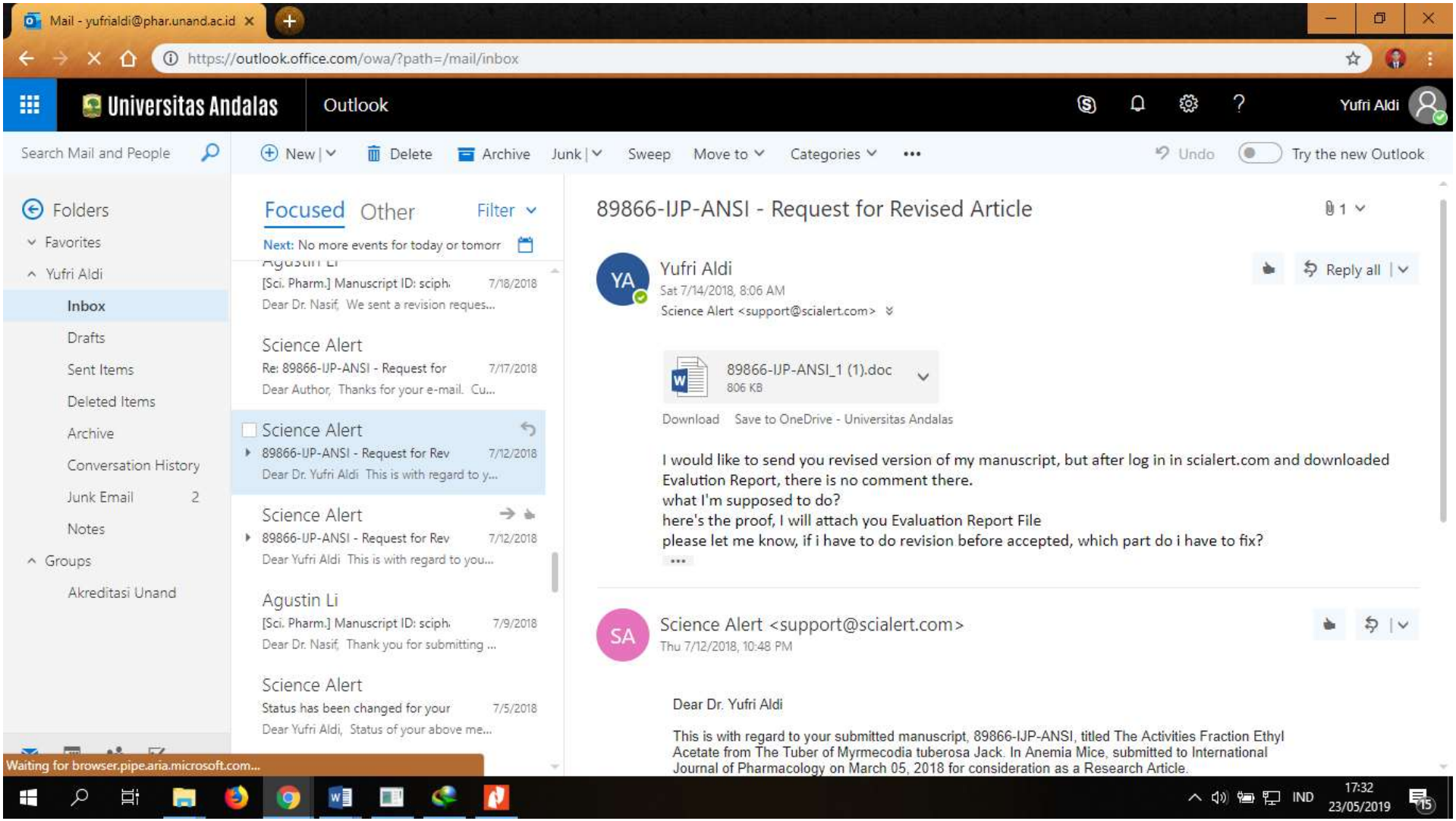
21. Status artikel berubah menjadi **"Manuscript assigned to Reviewer for Scientific Review"**



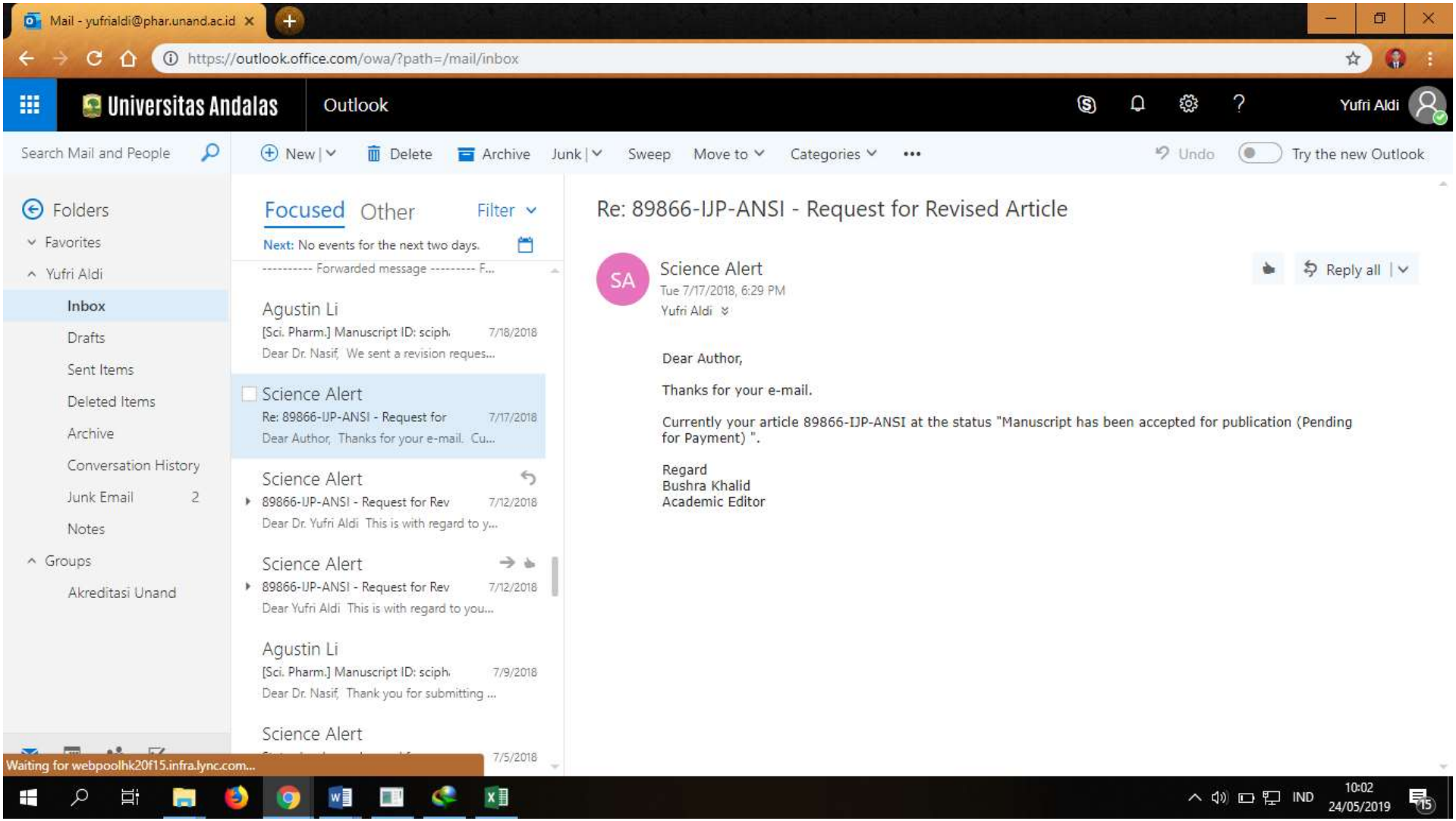
22. Status artikel berubah menjadi **"Accepted for publication after revision"**



23. Menyerahkan revisi terakhir sebelum dengan status jurnal terakhir **"Accepted after revision"**



24. Status artikel berubah menjadi **"Accepted for publication pending for payment"**



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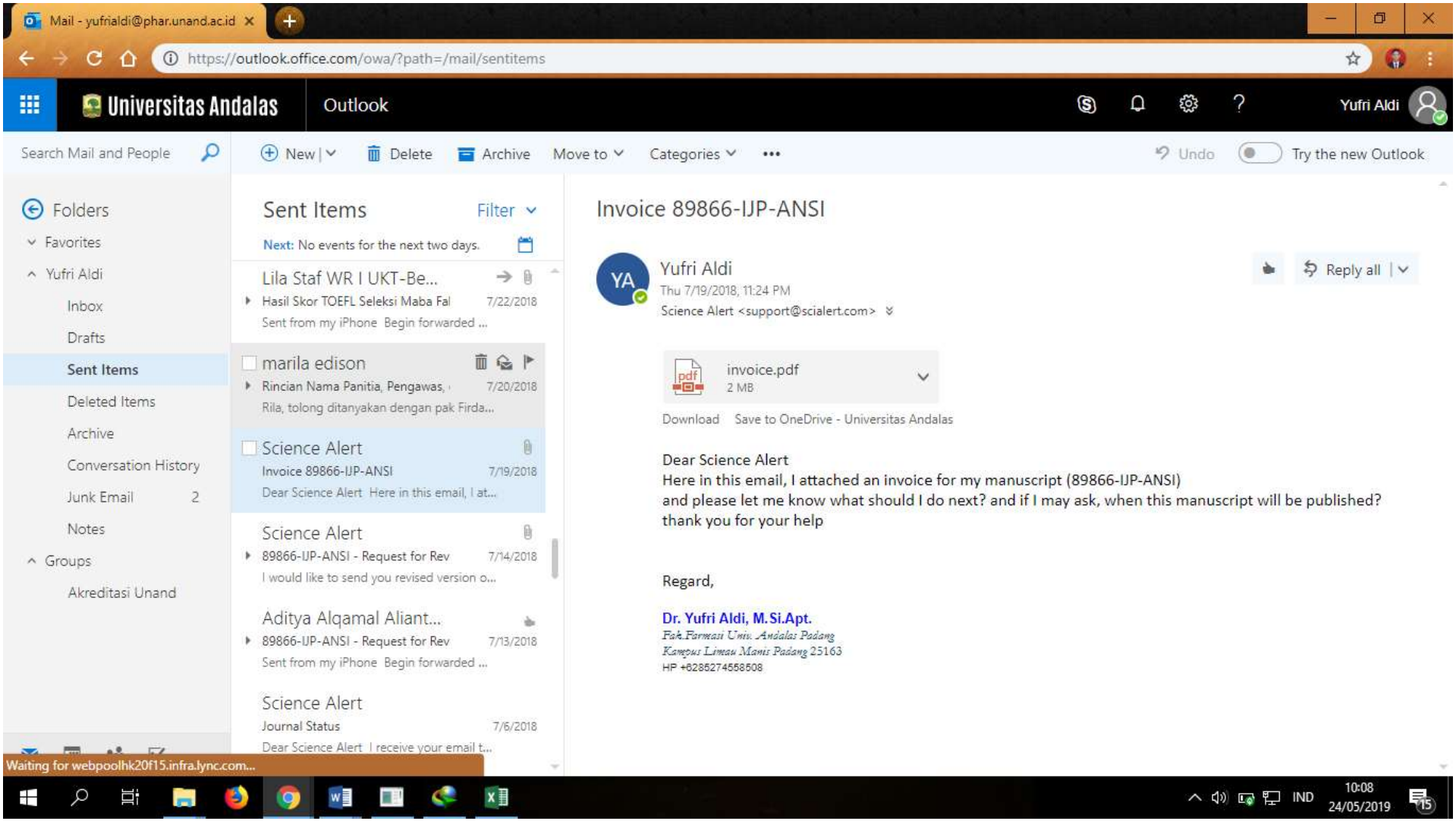

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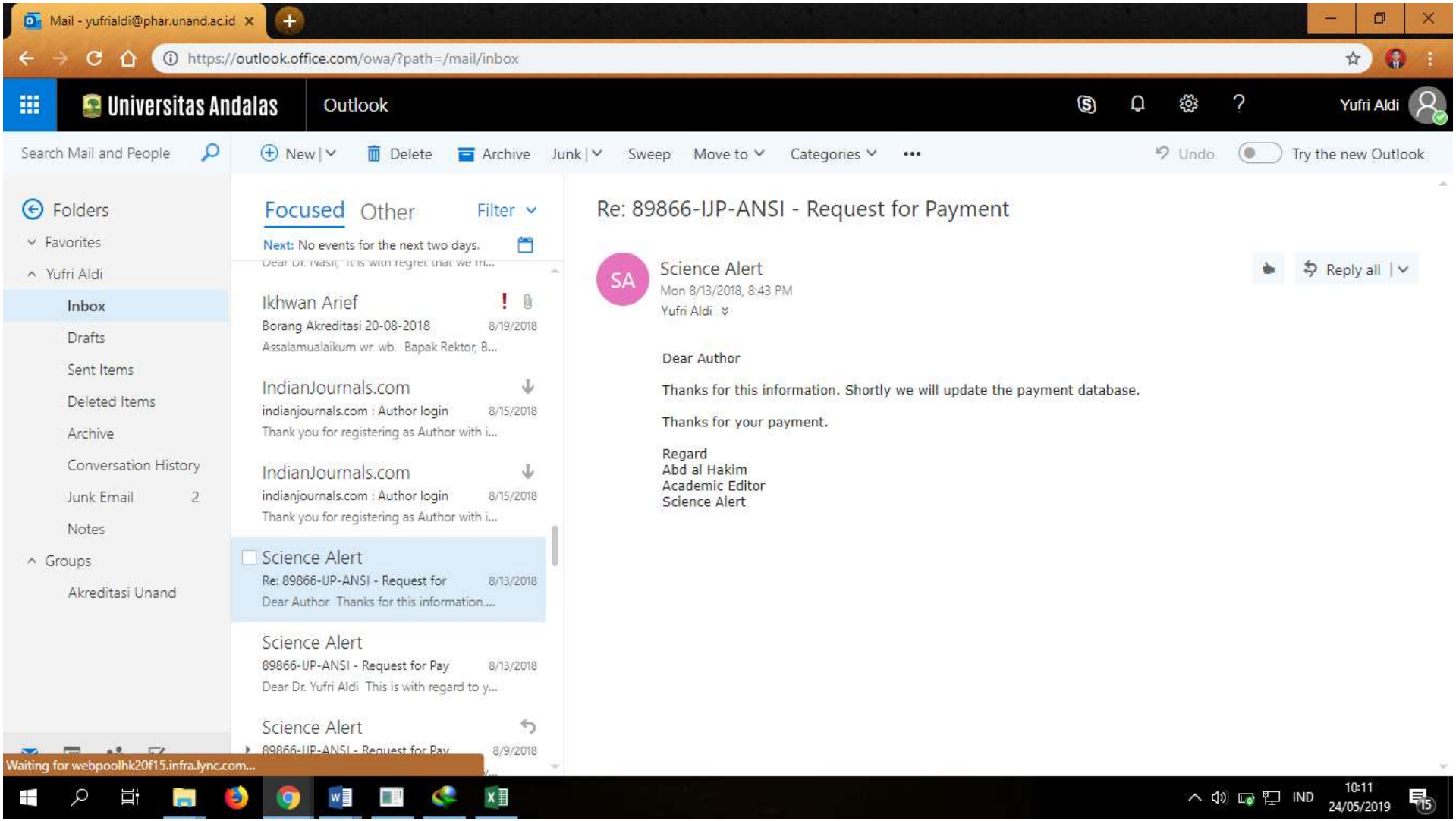

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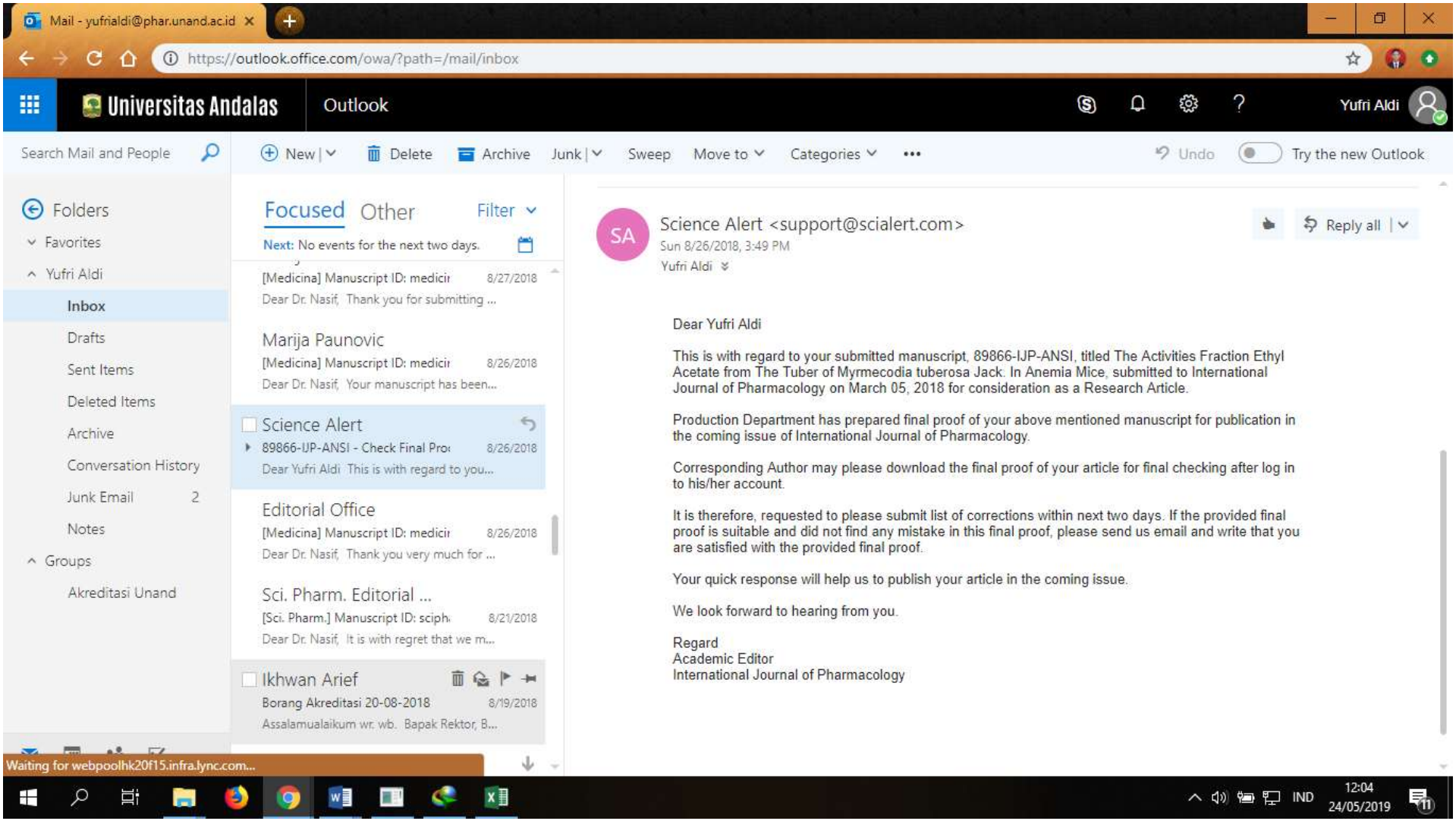

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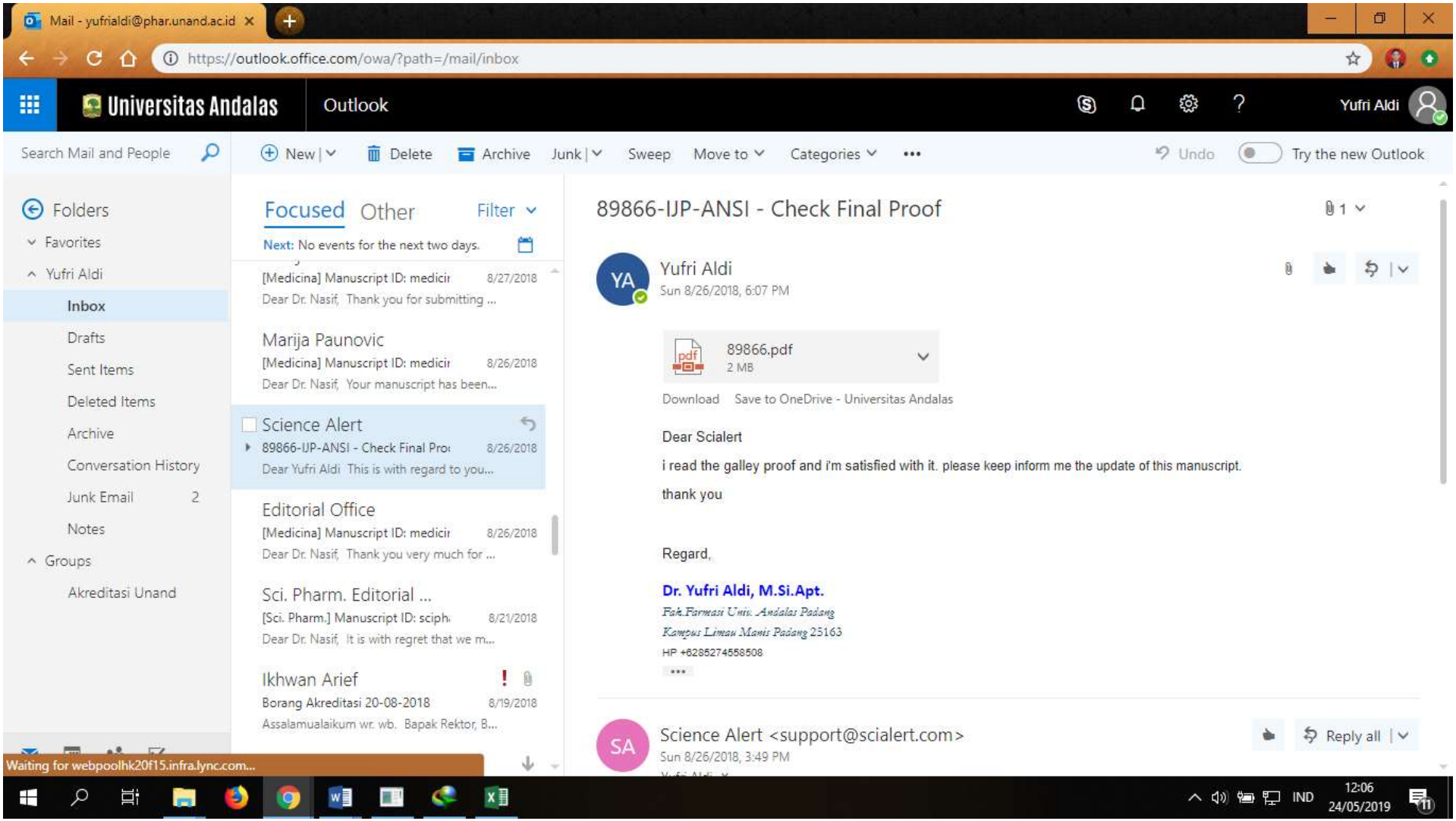

Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack. in Anemic Mice

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 classified as aplastic

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

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

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

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

The increase in reticulocyte count suggests that, as expected, chloramphenicol only caused reversible suppression of the bone marrow function and did not permanently damage its ability to produce erythropoietin[29]. On the contrary, the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. appears to stimulate reticulocyte production in the bone 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 7. A fresh “ant nest” tuber *Myrmecodia tuberosa* Jack.

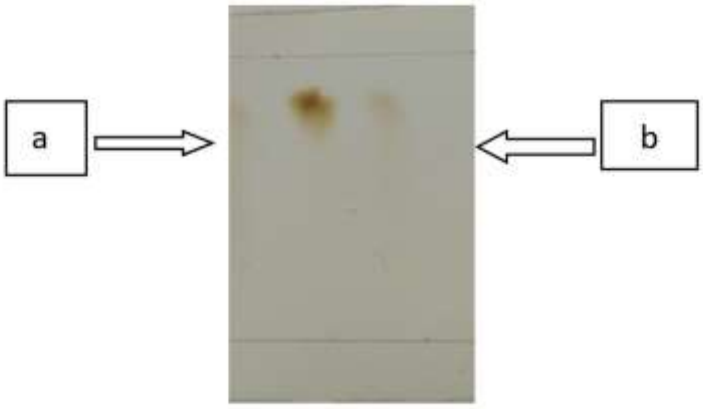

Figure 2. TLC profile of the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. under UV light (254 nm) using an eluent mixture of butanol: acetate acid: water (2:0.5:2.5). Note a = quercetin standard b = ethyl acetate fraction of *Myrmecodia tuberosa* Jack.

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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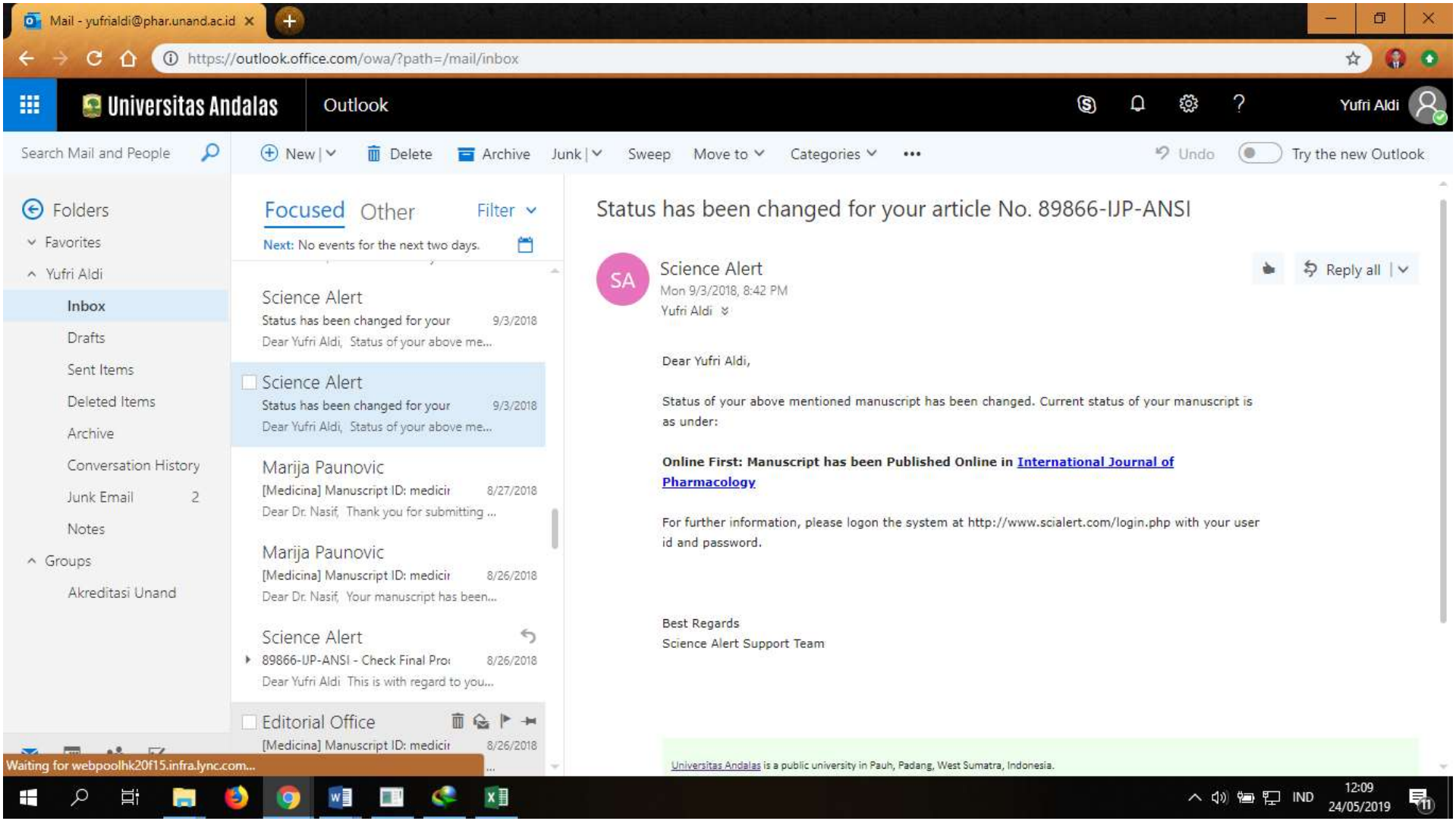

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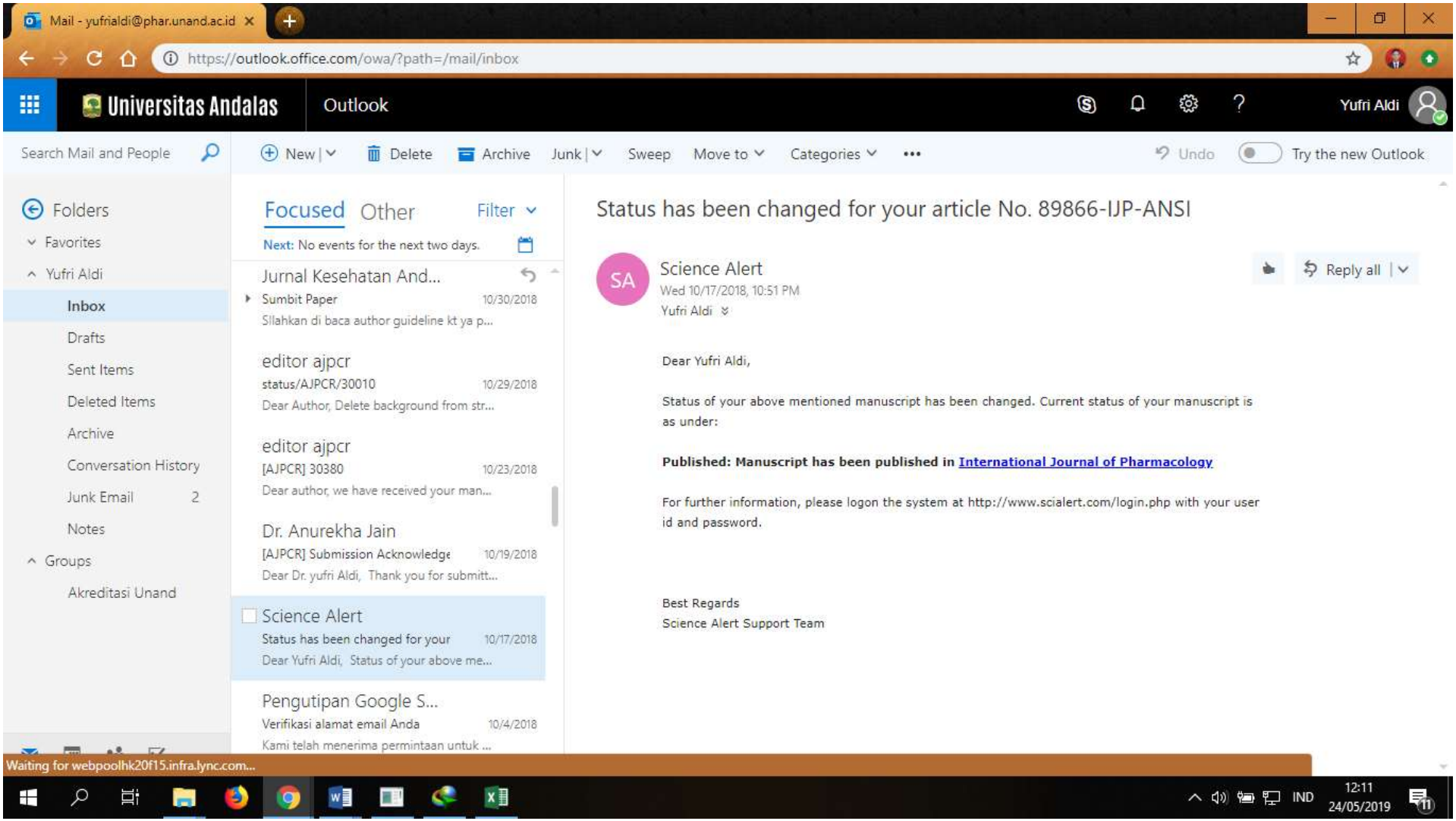

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Research Article
Ethyl Acetate Fraction Activities of *Myrmecodia tuberosa* Jack. in Anemic Mice

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Abstract

Background and Objective: 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 kg^{-1} b.wt., for 14 days then for next 14 days daily oral doses of 40 mg kg^{-1} b.wt., 63.2 mg kg^{-1} b.wt., or 100 mg kg^{-1} b.wt., 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:** About 40-63.2 mg kg^{-1} b.wt. doses of *Myrmecodia tuberosa* Jack. ethyl acetate fraction significantly increased the erythrocyte, reticulocyte and hemoglobin count and hematocrit from the 14th day ($p<0.01$). **Conclusion:** Ethyl acetate fraction of *Myrmecodia tuberosa* Jack. could have potential as an anemia treatment.

Key words: Anemia, erythrocyte, hematocrit, hemoglobin, *Myrmecodia tuberosa* jack., reticulocyte

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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 Sprague Dawley (SD) 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^{-1} b.wt., for a woman or 14 g dL^{-1} b.wt., 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 study 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.

MATERIALS 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 200 mg mL^{-1}.

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

Fig. 1: A fresh "ant nest" tuber *Myrmecodia tuberosa* Jack

Mark) spectrophotometer, erythrocyte pipette, hemocytometer and microscope (ZEISS).

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

Extraction and fractionation *Myrmecodia tuberosa* Jack: About 4 kg 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 400 g of powder which was placed in a dark macerator bottle with 4 L 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.

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.

Treatment of mice: About 130 mg kg^{-1} b.wt., 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, 63.2 and 100 mg kg^{-1} b.wt., 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 min, two drops discarded then the tip placed on a glass slide and covered with a coverslip. After 2-3 min 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 min for the dye to be absorbed by the blood cells. About 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: Five milliliter Drabkin solution was mixed with 20 µL blood and shaken in a tube until well mixed then set aside at room temperature for 3 min. 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 min. 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 Fig. 2.

Erythrocyte counts (million μL^{-1}) 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 kg^{-1} b.wt., 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 and 100 mg kg^{-1} b.wt., 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[14,24]. 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,

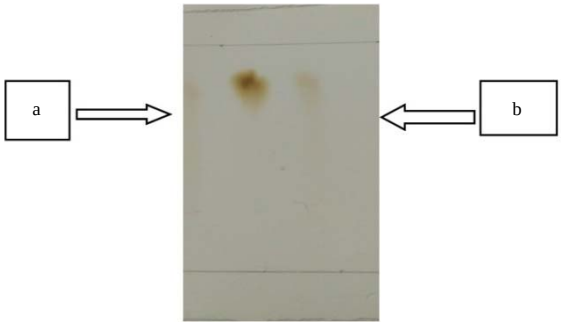

Fig. 2: TLC profile of the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. under UV light (254 nm) using an eluent mixture of butanol: acetate acid: water (2:0.5:2.5)
a: Quercetin standard, b: Ethyl acetate fraction of *Myrmecodia tuberosa* Jack

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

	Amount of erythrocyte (millions μL^{-1})			
Doses	Day 14	Day 21	Day 28	Average±SD
Positive control	4.39±0.19	4.83±0.20	5.25±0.14	4.82±0.40
Dose 40 mg kg^{-1} b.wt.	4.39±0.19	5.18±0.26	5.58±0.20	5.04±0.56
Dose 63.2 mg kg^{-1} b.wt.	4.41±0.13	5.59±0.36	5.86±0.26	5.29±0.70
Dose 100 mg kg^{-1} b.wt.	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

Sources	Type III sum of squares	df	Mean square	F-value	Significant
Doses	2.638	3	0.879	21.517	0.000
Duration	16.927	2	8.464	207.115	0.000
Doses and duration	1.099	6	0.183	4.483	0.001
Total	1599.179	60			

df: Degree of freedom

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

		Subset for alpha = 0.05		
Treatments	N	1	2	3
Doses				
Positive control	20	4.8213		
40 mg kg^{-1} b.wt.	20		5.0447	
63.2 mg kg^{-1} b.wt.	20			5.2880
100 mg kg^{-1} b.wt.	20			5.3500
Significant		1.0000	1.0000	0.4050
Duration				
14th day	20	4.4050		
21st day	20		5.3040	
28th day	20			5.6690
Significant		1.0000	1.0000	1.0000

occurs in mammals whenever hemoglobin level drops below 12 g dL^{-1} b.wt., for female and 14 g dL^{-1} b.wt., 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 classified as aplastic anemia. Anema aplastic is a deficiency of erythrocytes, reticulocytes, hemoglobin and hematocrit as a result of reduction of erythroblast cells being produced in the bone marrow[22,26].

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

The reticulocyte count for 14 days of ethyl acetate fraction administration is shown in Table 4. The increase is highly significant ($p<0.01$). The increase due to 40 mg kg^{-1} b.wt., and 63.2 and 100 mg kg^{-1} b.wt., was highly significantly different ($p<0.01$).

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

The increase in reticulocyte count suggested that, as expected, chloramphenicol only caused reversible suppression of the bone marrow function and did not permanently damage its ability to produce erythropoietin[27]. On the contrary, the ethyl acetate fraction of *Myrmecodia tuberosa* Jack. appears to stimulate reticulocyte production in the bone marrow[26]. 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[28,29].

The average content of hemoglobin (g dL^{-1} b.wt.,) 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 or 63.2 mg kg^{-1} b.wt., dose resulted in hemoglobin levels significantly higher than the positive control, the 100 mg kg^{-1} b.wt., dose did result in a significant increase ($p<0.05$).

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

	Amount of reticulocyte (millions μL^{-1})			
Doses	Day 14	Day 21	Day 28	Average±SD
Positive control	0.42±0.04	0.68±0.08	0.78±0.04	0.63±0.17
Dose 40 mg kg^{-1} b.wt.	0.48±0.08	0.76±0.11	0.86±0.09	0.70±0.19
Dose 63.2 mg kg^{-1} b.wt.	0.44±0.05	0.78±0.08	0.96±0.11	0.73±0.24
Dose 100 mg kg^{-1} b.wt.	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	

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

Sources	Type III sum of squares	df	Mean square	F	Significant
Doses	0.815	3	0.272	30.191	0.000
Duration	3.194	2	1.597	177.463	0.000
Doses and Duration	0.588	6	0.098	10.895	0.000
Total	38.630	60			

df: Degree of Freedom

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

		Subset for alpha = 0.05		
Treatments	N	1	2	3
Doses				
Positive control	20	0.6267		
40 mg kg^{-1} b.wt.	20		0.7000	
63.2 mg kg^{-1} b.wt.	20		0.7267	
100 mg kg^{-1} b.wt.	20			0.9400
Significant		1.0000	0.4450	1.0000
Duration				
14th day	20	0.4400		
21st day	20		0.8100	
28th day	20			0.9950
Significant		1.0000	1.0000	1.0000

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

	Content of hemoglobin (g dL^{-1})			
Doses	Day 14	Day 21	Day 28	Average±SD
Positive control	11.93±1.13	14.37±0.65	15.67±0.69	13.99±1.79
Dose 40 mg kg^{-1} b.wt.	11.98±0.55	14.96±0.58	15.77±0.65	14.24±1.77
Dose 63.2 mg kg^{-1} b.wt.	12.10±0.59	15.02±1.47	15.91±1.78	14.34±2.12
Dose 100 mg kg^{-1} b.wt.	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

Sources	Type III sum of squares	df	Mean square	F	Significant
Doses	30.103	3	10.034	8.403	0.000
Duration	206.559	2	103.279	86.484	0.000
Doses and duration	12.834	6	2.139	1.791	0.121
Total	13082.989	60			

df: Degree of freedom

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

		Subset for alpha = 0.05		
Treatments	N	1	2	3
Positive control	20	13.9887		
40 mg kg^{-1} b.wt.	20	14.2380		
63.2 mg kg^{-1} b.wt.	20	14.3440		
100 mg kg^{-1} b.wt.	20		15.7987	
Significant		0.4070	1.0000	
Duration				
14th day	20	12.0375		
21st day	20		15.3515	
28th day	20			16.3880
Significant		1.0000	1.0000	1.0000

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, 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

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

	Value of hematocrit (%)			
Doses	Day 14	Day 21	Day 28	Average±SD
Positive control	41.9±1.75	44.2±1.48	45.6±1.48	43.9±2.09
Dose 40 mg kg^{-1} b.wt.	43.8±2.49	45.0±1.58	46.9±2.22	45.2±2.37
Dose 63.2 mg kg^{-1} b.wt.	43.2±2.59	45.2±1.95	47.4±1.14	453±2.56
Dose 100 mg kg^{-1} b.wt.	43.5±2.57	46.7±2.73	49.2±2.92	46.5±3.50
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

Sources	Type III sum of squares	df	Mean square	F-value	Significant
Doses	49.483	3	16.494	3.622	0.019
Duration	174.408	2	87.204	19.148	0.000
Doses and duration	10.692	6	1.782	0.391	0.881
Total	123126.000	60			

df: Degree of freedom

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

		Subset for alpha = 0.05		
Treatments	N	1	2	3
Doses				
The positive control	20	43.900		
40 mg kg^{-1} b.wt.	20	45.233	45.233	
63.2 mg kg^{-1} b.wt.	20	45.267	45.267	
100 mg kg^{-1} b.wt.	20		46.467	
Significant		0.103	0.141	
Duration				
14th day	20	43.100		
21st day	20		45.275	
28th day	20			47.275
Significant		1.000	1.000	1.0000

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, 63.2 and 100 mg kg^{-1} b.wt., 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 suggested 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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