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Immunomodulatory Activity of *Allium sativum*, C*urcuma mangga*, and *Acorus calamus* Combination Nanoparticle on Mice Leukocytes Profile

Bayyinatul Muchtaromah[1*], Nur Izzah Analisa[2], Mujahidin Ahmad[3], Prilya Dewi Fitriasari[4], Soraya Habibi[5], Alfiah Hayati[6], Ely Nuril Fajriyah[7]

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[1,7]Magister Program of Biology, Faculty of Science and Technology, UIN Maulana Malik Ibrahim, Jl. Gajayana No.50, Malang, East Java, Indonesia, 65144, [2,3,4]Department of Biology, Faculty of Science and Technology, UIN Maulana Malik Ibrahim, Jl. Gajayana No.50, Malang, East Java, Indonesia, 65144, [5]Department of Biology, Faculty of Math and Science, Universitas Terbuka, Jl. Mayjen Sungkono No.9, Bumiayu, Malang, East Java, Indonesia, 65135, [6]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Jl. Mayjen Prof. Dr. Moestopo No.47, Surabaya, East Java, Indonesia, 60132

email:
*[1]*bayyinatul@bio.uin-malang.ac.i,*
[2]*analisaizzah@gmail.com*
[3]*mujahidin@bio.uin-malang.ac.id*
[4]*prilyadewi@bio.uin-malang.ac.id*
[5]*soraya@ut.ac.id*
[6]*alfiah-h@fst.unair.ac.id*
[7]*elynuril15@gmail.com*

**Corresponding author*

Abstract. *Immunomodulators are substances acting as stimulants or suppressants against the immune system, either to specific or non-specific activity. The non-specific through the production of leukocytes as the first response in fighting against an attacking antigen. Meanwhile, the specific, hrough recognition of antigens by lymphocytes during reattack. This research aimed to determine the immunomodulatory activity of the extracts combination nanoparticles of Allium sativum, Curcuma mangga, and Acorus calamus on the leukocyte profile of mice. This study used 5 treatments and 6 repetitions. The treatment groups were K- (untreated group), P1 (extracts combination nanoparticle dose of 25 mg/kg), P2 (extracts combination nanoparticle dose of 50 mg / kg), P3 (subur kandungan herbal medicine dose of 75 mg / kg), and P4 (Clomiphene citrate dose of 0.9 mg / kgBW). The parameters used included the total number of leukocytes and their differential value. The data that met the parametric assumptions, such as normally distributed and homogeneous were examined using the* One Way ANOVA *test, and when there was a significant difference, it was processed with the* Duncan *assessment. While those that did not meet the assumptions were evaluated using a non-parametric analysis. The statistical results showed that administration of extracts combination nanoparticles of A. sativum, C. mangga, and A. calamus at doses of 25 mg/kg and 50 mg/kg were able to suppress the inflammatory reaction by decreasing the total number of leukocytes. However, the differential leukocyte count was able to maintained or modulate immune system, indicating by the percentage of neutrophil, basophil, and eosinophil in the normal range. At a dose of 50 mg/kg, decreased the percentage of lymphocytes, while for monocytes, all dosage ranges were able to increase their number.*

Keywords: *Capsicum annuum, characterization, chilli, dual culture, PGPR.*

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INTRODUCTION

Immunomodulators are substances used in restoring imbalances in the immune system, either to specific or non-specific activity (Wahyuni et al., 2019). One of the cells that play a role in the non-specific immune system is the leukocyte, which is a blood component that maintains the body's homeostasis and is the first protector in fighting against

attacking antigens (Al-Dulaimi et al., 2018). Therefore, analysis of the leukocytes structure is important in determining the condition of an individual (Putzu & Ruberto, 2013). The number and the percentage of differential leukocytes are considered key components of the immune system because they are the main builders of the response produced (Shabbir et al., 2016). There are two types of leukocytes, namely granulocytes and agranulocytes. The granulocytes include neutrophils, basophils, and eosinophils, while agranulocytes consist of lymphocytes and monocytes (Putzu & Ruberto, 2013).

Paraimmunity is the non-specific immune system fights against antigen.The substance that induced it, known as an inductor, has very little antigenic action. Therefore, some of them act as mitogens in increasing cell proliferation that play a role in the immune system (Sadsoeitoeboen et al., 2019). There are two types of immunomodulators depending on their effect, namely immunostimulators and immunosuppressors. Immunostimulators are substances that provide an effect to increase the immune system in both healthy and unhealthy conditions, while immunosuppressors act to suppress it (Saroj et al., 2012).

Various ingredients from plants modulate immune activity in a diverse manner (Zmora et al., 2017). In different parts of the world, plant extracts have been widely investigated as immunomodulators including the *Acorus calamus* (Abood, 2017), as they contain varieties of active compounds. One of the plant extracts is. In Indonesia, plant extracts are usually used in traditional medicine and are known as jamu (Beers, 2012). Also, they have been used for centuries to treat various diseases and maintain good health (Amalia & Aprianingsih, 2017).

Indonesia has diverse plants, around 2500 species have medicinal potential (Elfahmi et al., 2014). One of the areas that still implement traditional medicine culture is Madura. The herb that quite popular in this region is the subur kandungan (Satriyati, 2017). It is produced by PT. Ribkah Maryam Jokotole Bangkalan, Madura, and consists of three main ingredients, namely *Allium sativum* (Garlic), *Curcuma mangga* (Turmeric), and *Acorus calamus* (Sweet Flag) (Muchtaromah et al, 2017).

Allium sativum has many benefits, such as antimicrobial, antiviral, and immunomodulatory. It is rich in organosulfur, tannins, and glycosides which have biological and pharmacological effects (Ali & Ibrahim, 2013). According to Rosdianto et al (2016), giving fertility herbs containing *A. sativum*, was able to increase the total number of white rat leukocytes by giving a dose of 50.4 mg for 15 days. *C. mangga* contains curcuminoids, glycosides, and anthrax. It has also been reported to have anti-inflammatory, immunomodulatory, and no inhibitory activities (Kamazeri et al, 2012). *A. calamus* contains alkaloids, triterpenoids, and essential oils which are useful as anti-inflammatory, immunomodulatory, and antifungal (Muchtaromah et al, 2017). This research aimed to determine the immunomodulatory activity of the extracts combination nanoparticle of *A. sativum, C. mangga,* and *A. calamus* coated chitosan on the leukocyte profile of mice.

MATERIALS AND METHODS

This was experimental research. The extraction of active compounds from the combination of *A. sativum*, *C. mangga,* and *A. calamus* was carried out using the maceration method with 70% ethanol as the solvent. The extraction results were then synthesized into nanoparticles coated with chitosan using the

ionic gelation method. The immunomodulatory activity tests on leukocyte profiles were carried out manually using a haemacytometer and peripheral blood smear slides. The total number of leukocytes is one of the key components of the immune system. The existence of the immune system is strongly influenced by leukocytes, as leukocytes are able to recognize foreign objects and build an immune response in the body (Shabbir, et al., 2016; Sultana, et al., 2011). Sugiharto et al (2015) stated that the total number of leukocytes and their derivatives was able to provide an overview of the health status of animals.

The materials used in this research were simplicias of *A. sativum*, *C. mangga,* and *A. calamus* simplicia obtained from UPT. Materia Medika Batu. The samples for nanoparticles synthesis were obtained from Merck, while female mice were collected from the Animal Experimental Development Unit (UPHP) Jl. Soekarno Hatta Malang City. The research procedure was approved by the Health Research Ethics Committee (KEPK), Faculty of Science and Technology, State Islamic University of Maulana Malik Ibrahim Malang (approval reference number: 021/EC/KEP-FST/2021).

The extraction process commenced with *A. sativum*, *C. mangga*, and *A. calamus* simplicia (36%: 36%: 28%) macerated using 70% ethanol (Merck), soaked for 24 hours, and filtered. The maceration process was repeated three times to obtain a clear filtrate, which was concentrated by a rotary evaporator at 50˚C.

The synthesis of extracts combination nanoparticles of *A. sativum*, *C. mangga*, and *A. calamus* coated chitosan was following the modified procedure of Pakki et al (2016). Then, 0.5% glacial acetic acid (AAG) was dissolved in 100 ml of distilled water and homogenized using a stirrer, added with 0.5% chitosan, and blended again. In addition, 0.5% STTP solution was dissolved in 20 ml of distilled water and homogenized at 1000 rpm for 10 mins. Then the two solutions were mixed at 1000 rpm for 10 mins. After that, 0.1 g of the combined extract of *A. sativum*, *C. mangga*, and *A. calamus* was added, then blended at 3000 rpm for 30 mins. Next, 1 ml of tween 80 was added and homogenized at 10000 rpm for 90 mins and sonicated at a frequency of 20 kHz with an amplitude of 90% for 90 mins. The obtained solution was poured into a 15 ml eppendorf tube and centrifuged at 5000 rpm for 30 mins to produce pellets. The yields were aerated or placed in the freezer for 24 hours, and allowed lyophilization, to produce nanoparticle powder.

Mus musculus Balb-C strain aged about 2-3 months with a bodyweight of 20-25 g. The mice must be active, have soft and strong hair, indicated by not easily falling out during acclimatization in the Experimental Animal Laboratory, Biology Study Program, Faculty of Science and Technology, State Islamic University of Maulana Malik Ibrahim Malang for two weeks. The mice were then divided into several treatment groups, namely K- (negative control/distilled water), P1 (25 mg/kg of extracts combination nanoparticle *A sativum*, *C. mangga,* and *A. calamus* coated chitosan), P2 (50 mg/kg of extracts combination nanoparticle of *A. sativum*, *C. mangga,* and *A. calamus* coated chitosan), P3 (75 mg/kg of subur kandungan herbal medicine), and P4 (0.9 mg/kg of clomiphene citrate). The treatments were carried out for 15 days. This refers to the research of Rosdianto et al (2016), that the administration of fertility herbs in mice can modulate leukocytes within 3 times the estrous cycle (15 days). The *M. musculus* was fed with BRI pellets and given ad-libitum in the form of mineral water.

The total leukocyte count was carried

out on blood samples taken using the tail vein sampling method (Paim et al., 2011). And was aspirated using a leukocyte Thoma pipette up to line 0.5, then followed by a Turk solution (to lyse erythrocytes) up to line 11. The mixture in the pipette was homogenized by shaking following an eight-like motion, while the solution on the tip which was stable was thrown away first before being used. Then, the homogeneous mixture was dropped into the counting room slide by gluing the tip of the pipette between the bottom of the counting room slide which has been covered by a glass. The samples were observed using a microscope with a magnification of 100x to 400x. Leukocytes were observed as dark purple in color (Bjorner & Zhu, 2019). Then, the leukocyte calculations were carried out in the four large boxes of the counting room slide with the formula for cells x 20 (factor dilution) x 10: 4 (number of boxes in mm^3) (Dillasamola et al., 2019).

The leukocytes calculation was conducted by dripping the blood sample into the object-glass provided, drained using another vessel, and finally dried. Blood samples were fixed in absolute methanol for 3-5 mins and allowed to dry. After the fixation stage, the blood smear was soaked in Giemsa staining for 1 min and was washed using flowing distilled water to remove the unnecessary parts slowly. The smears were dried and observed using the lowest to the largest magnification of 1000x. The types of leukocytes were observed until the total reached 100 cells. Next, the percentage calculation of leukocytes was carried out to determine the absolute value of each by multiplying their % with the total number of these cells and 100% (Bjorner & Zhu, 2019).

The research data were analyzed by the normality and homogeneity test. When both satisfied the parametric assessments, it was followed by the analysis of variance (ANOVA) and Duncan's test. However, when they did not, they were analyzed using the non-parametric. All tests used the SPSS 16.0 program (SPSS Inc., USA). The data were stated to be significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

Number of Leukocyte

The non-specific immune system involved in this research was the leukocyte, the main activator unit (Olaniyan et al., 2013). It plays an important role in protecting the body from various infections. These leukocytes also act as intermediaries between the non-specific and the specific immune system (Fathima & Khanum, 2017). Therefore, leukocytes used as a parameter in measuring the level of individuals' immunity. Leukocyte parameters in this research were carried out by measuring their total number in each treatment group.

The total number of leukocytes obtained from each treatment showed a different mean total number of leukocytes and was still in the normal range. These include group K- (8133.3 ± 1809.6), P1 (6866.7 ± 1170.8), P2 (4600.0 ± 1166.2), P3 (4200.0 ± 1725.1), and P4 (6766.7 ± 1592.1) (Table 1).

The statistical results of the total number of leukocytes using One Way Anova were 0.001 ($p < 0.05$), this indicated a significant difference. Duncan's test showed that there was an increase in the total number of leukocytes in the control group (K-) when compared to the other treatments. The mean total number of leukocytes in the control group (K-) was 8133.3 cells / mm^3. The increase that occurred in the control group (K-) was still in the normal range. While the treatment groups P1, P2, P3, and P4 experienced a decrease when compared to the control (K-), which was still within the normal range (Figure 1). According to

Weiss & Wardrop (2010), the total number of leukocytes in normal mice ranges from 2000-10000 cells / mm^3. This showed that the treatment suppressed the inflammatory activity.

The decrease in the total number of leukocytes that occurred in the treatment groups other than the K-, was due to the administration of extracts combination nanoparticles of *A. sativum*, *C. mangga,* and *A. calamus*, which contained active compounds, such as flavonoids, saponins, alkaloids, etcetera. These compounds modulate the immune system by enhancing or suppressing its excess. This is based on the study of Zhang et al (2017), which stated that flavonoids contained in a plant sometimes do not work synergistically, because these compounds have immunostimulant and immunosuppressant effects. Nanang et al (2013) added that plant steroid compounds also decreased the total number of leukocytes and have the potential as an anti-inflammation.

Table 1. Immunomodulatory activity of extracts combination nanoparticles of *A. sativum, C. mangga,* and *A. calamus* on the total number of leukocyte in mice

Treatment	Total Number of Leukocyte (cell/mm^3)
	Mean ± SD
K-	8133.3 ± 1809.6*
P1	6866.7 ± 1170.8
P2	4600.0 ± 1166.2*
P3	4200.0 ± 1725.1*
P4	6766.7 ± 1592.1

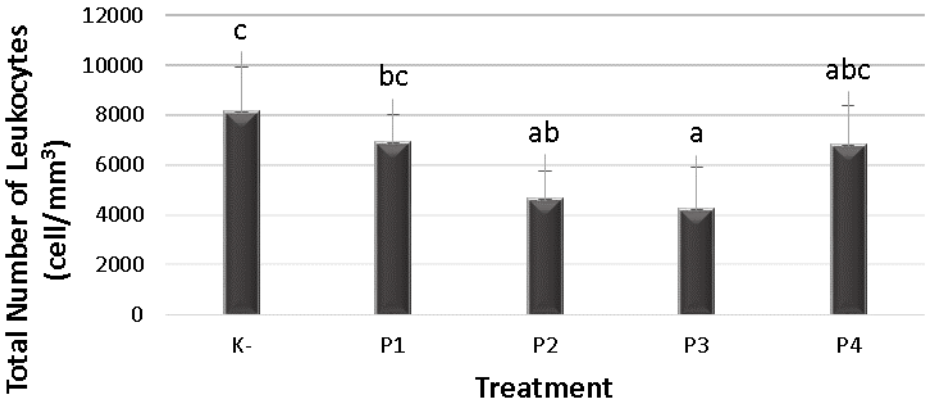

Figure 1. The mean of total number of leukocyte mice

Differential Leukocytes

The types of leukocytes and their variables are the blood components that describe the immunity health status of animals (Sugiharto, et al., 2015). This research was conducted based on the observation and calculation of the percentage related to the types of leukocytes, including granulocyte and agranulocyte. The observed granulocytes were neutrophils, basophils, and eosinophils, while the agranulocytes were lymphocytes and monocytes.

The results on differential leukocytes showed that the extracts combination nanopar-

ticle of *A. sativum*, *C. mangga,* and *A. calamus* was significantly different in the percentage of basophil, lymphocyte, and monocyte. Meanwhile, the neutrophil and eosinophil did not show any significant differences in each group. The mean percentages of neutrophil, basophil, and eosinophil were found to be in the normal range (Table 2). According to Nugroho (2018), the number of leukocytes in the normal mice were around 6.7%-37.2% (neutrophils), 0-1.5% (basophils), 0.9%-3.8%. (eosinophils), 63-75% (lymphocytes) and 0.7-2.6% (monocytes).

The results on neutrophil, basophil, and eosinophil above indicated that the mice used were normal/healthy and did not experience inflammation or allergies. The percentage of neutrophils that tend to be stable and maintained within normal limits indicated that the mice were normal (Weiss & Wardrop, 2010). The basophil which was still in the normal range showed that there was no inflammation in the animal model, and proved that the treatment did not cause allergic reactions (Muhsin, 2017). According to Jatmiko (2015), the absence of eosinophils in a stable state indicated the absence of sick mice due to parasite infection.

The statistical results on the percentage of lymphocytes showed that there was a significant difference between the clomiphene citrate group at a dose of 0.9 mg/kgBW (45%), which was significantly different compared to other treatments. Meanwhile, the P1, P2, and P3 groups were not significantly different from the control. The mean lymphocyte percentage in each group of K-, P1, and P3 were still in the range of normal. While P2 showed lower the percentage of lymphocytes than normal, however, this was not significantly different from the K-, P1, and P3 groups (Figure 2).

The little decrease of lymphocytes in group P2 was assumed to be caused by the active compounds of *A. sativum*, *C. mangga,* and *A. calamus* in the form of flavonoids, saponins, phenols, alkaloids, etc. Wang et al (2007) stated that the amphiphilic compounds contained in an herbal extract (flavonoids and saponins) are known to increase the P27KIP protein level, which plays an important role in regulating cell proliferation in the G0/G1 phase by inhibiting the G1 Cyclin-CDK complex. The presence of inhibition in this complex causes the Cyclin-CDK complex not to be activated, therefore, the cell cycle does not continue to the next phase, and the cell proliferation process stopped. This is also supported by the statement of Pavlopa et al. (2015) that many flavonoids can suppress in vitro mitogen-induced lymphocyte proliferation. So, there is a decrease in IL-2 and IFNγ, as the activity is inhibited.

The data on the percentage of monocytes were known not to be homogeneous, therefore, was continued with the non-parametric Brown-Forsythe test (Field, 2009). The results showed the value of $p = 0.00$ ($p < 0.05$). This indicated that there was a difference in the mean percentage of monocytes in each treatment group, therefore, it was continued with the non-parametric Games-Howell test.

The results of the non-parametric Games-Howell test showed that the mean percentage of monocytes in the clomiphene citrate group at a dose of 0.9 mg/kgBW (45%), was significantly different from the control and other treatments. Meanwhile, the P1, P2, and P3 groups ere not significantly different-from the control group. The mean percentage of monocytes in each group of K-, P1, P2, P3, and P4 showed an increase from their normal levels (Figure 2).

This is due to the flavonoids that activate lymph to increase monocyte production. These compounds have water-soluble characteristics and function as anti-microbial, an-

ti-viral, and immunostimulants. In addition, substances containing flavonoids also increase phagocytic activity (Syahida et al., 2013). This is caused by flavonoid compounds activating the T cells to produce lymphokines, like IL-2 and IL-10 which then stimulate phagocytic cells to carry out their activity (Nugroho, 2012; Al Osaj, 2013).

Table 2. Immunomodulatory activity of extracts combination nanoparticles of *A. sativum, C. mangga* and *A. calamus* on differential leukocyte of mice

Treatment	Average of Differential Leukocytes Percentage (%) ± SD				
	Neutrophil	Basophil	Eosinophil	Lymphocyte	Monocyte
K-	20.0 ± 8.0	1.2 ± 0.8*	1.5 ± 1.2	68.3 ± 6.3	9.0 ± 7.5
P1	22.8 ± 5.5	0.8 ± 0.7	1.8 ± 1.2	66.0 ± 8.0	9.0 ± 6.4
P2	19.2 ± 9.0	0.3 ± 0.5*	2.7 ± 0.8	62.2 ± 8.0	15.7 ± 8.7
P3	23.9 ± 7.9	0.2 ± 0.4*	2.5 ± 1.0	68.3 ± 7.6	5.2 ± 1.2
P4	21.2 ± 12.9	1.0 ± 0.6*	2.5 ± 1.6	45.3 ± 7.2*	30.0 ± 7.5*

*)significant at p<0,05

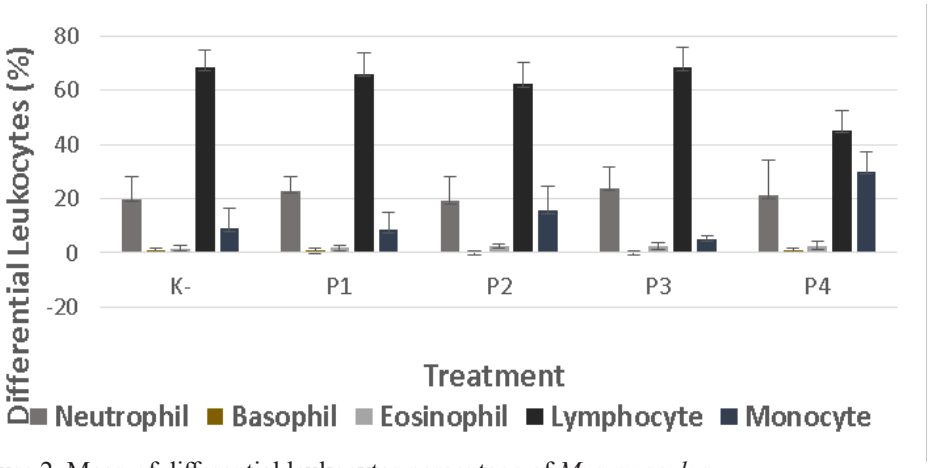

Figure 2. Mean of differential leukocytes percentage of *Mus musculus*

The types of leukocytes in this research were also presented in the form of microscopic images. Based on observations, it was known that neutrophils have a bluish-purple nucleus, which is curved shaped, segmented, and consists of more than one circular lobe. The neutrophil diameter reaches about 12.6 µm (Figure 3a). Basophil appears to have a purplish nucleus with granules in the cytoplasm, clustered in the middle, appeared unsegmented, and its cell diameter was found to be 15.5 µm (Figure 3b).

Eosinophil observation indicates a two-lobe nucleus, granules in the cytoplasm, appeared darker in color compared to neutrophils. The diameter of the eosinophils is known to be around 12 µm (Figure 3c). Lymphocytes have a dark purple single nucleus, not lobulated, almost covered with cytoplasm, with a cell diameter of about 6.4 µm (Figure 3d), While monocyte appeared to have a dark purple, hollow, slightly curved single nucleus, with a cell diameter of about 17.8 µm (Figure 3e).

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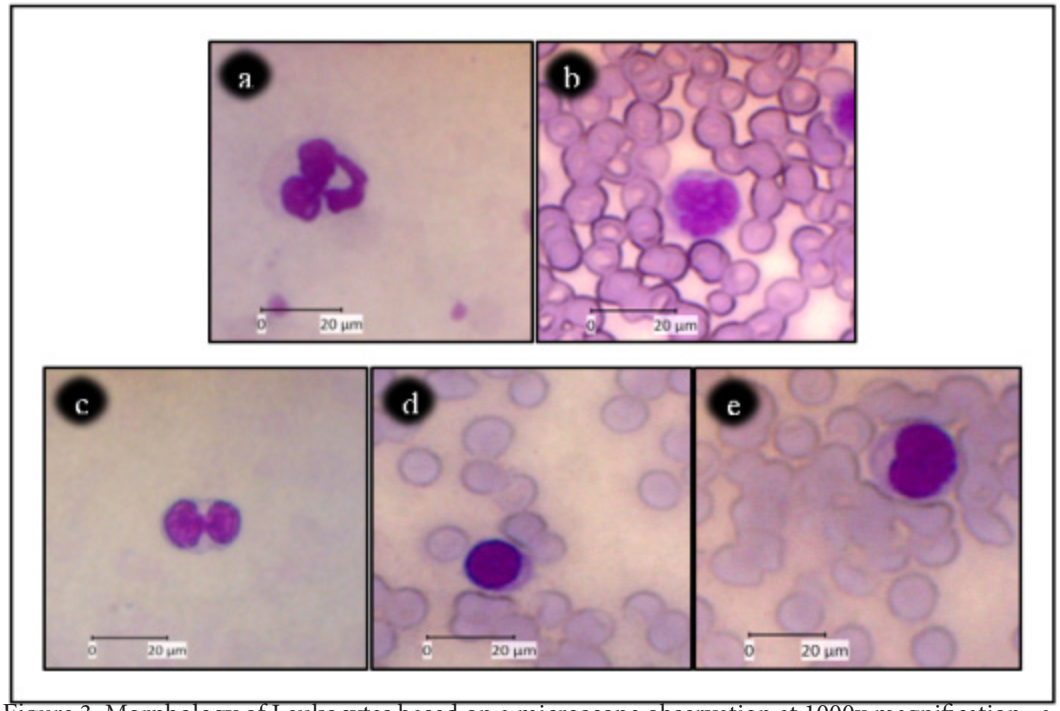

Figure 3. Morphology of Leukocytes based on a microscope observation at 1000x magnification: a. neutrophil b. basophil c. eosinophil d. lympochyte e. monocyte

CONCLUSION

The extracts of combination nanoparticles of *A. sativum*, *C. mangga* and *A. calamus* was able to suppress the inflammatory reaction by decreasing the total number of leukocytes and lymphocytes. In the percentage of neutrophil, basophil, and eosinophil, the treatment was able to maintain their numbers in the normal range. Meanwhile, in the percentage of monocyte, the treatment was able to increase its number in all dosage ranges.

AUTHOR CONTRIBUTION

B.M. made study conception design and supervised all the process, N.I.A. carried out research, collected and analyzed the data, as well as wrote the manuscript, M.A supervised all the process, P.D.F. proofread and edited manuscript, S.H. and A.H. reviewed the result of data analysis and edited manuscript, E. N. F collected the data.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

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