RESEARCH ARTICLE | JANUARY 30 2025

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AIP Conf. Proc. 3186, 020015 (2025)
https://doi.org/10.1063/5.0235708

The Effect of *Allium sativum, Curcuma mangga,* and *Acorus calamus* Nanoparticles to Profile of Mice Leukocytes Induced by Cisplatin

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Abstract. The induction of cisplatin can increase the production of *reactive oxygen species* (ROS) and cause an imbalance in the immune system. This condition can be overcome with use of *A. sativum, C. mangga*, and *A. calamus*. That plants contain several active compounds to improve the function of the immune system, including leukocytes. This study aimed to know the effect of nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* on the total cell leukocyte and total leukocyte types (neutrophil, basophil, eosinophil, lymphocyte, and monocyte) in mice that induced by cisplatin. This study used the Complete Random Design (CRD) with 5 treatments and 6 replicates. The examination of the total leukocyte used a hemocytometer and the total leukocyte types used a blood smear. The results showed that there was an effect of giving nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* on the total leukocyte, neutrophils, and lymphocytes mice and there was no effect of giving nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* to basophils and eosinophils mice that are induced by cisplatin. The dose of nanoparticles extracts *A. sativum*, *C. mangga*, and *A. calamus* 25 mg/kgBW and 50 mg/kgBW had a significant effect compared to the other treatment groups.

INTRODUCTION

Induction of cisplatin can cause infertility through cessation of primordial follicle regeneration, failure of ovarian development, and decreased anti-Mullerian hormone. The occurrence of infertility due to ovarian damage and dysfunction can increase free radicals [1]. The reactive nature of free radicals causes damage to cell components, such as proteins, lipids, and DNA, and causes mutations so that the immune system experiences an imbalance. The body's physiological response to overcome this is to activate the stress response in the form of glucocorticoid hormones which are regulated by cortisol produced by the Hypothalamus-Pituitary-Adrenal (HPA) axis to the activity and function of leukocytes. Leukocytes will die, the immune system will decrease, and cause reduced production of lymphocytes, eosinophils, monocytes, and basophils [2].

This condition of immune imbalance can be overcome by the use of herbal fertile content consisting of three main ingredients, namely *Allium sativum, Curcuma mangga*, and *Acorus calamus*. These three plants are efficacious to overcome infertility and improve immune system function [3]. The combination of that plants can form several kinds of activities, namely immunomodulators, antioxidants, and antimicrobials. In an in vivo study, it was shown that the use of extracts of *A. sativum*, *C. mangga*, and *A. calamus* could increase the fertility of female mice because of the complex activity relationship between reproductive hormones and the body's immune system [1]. Whereas in previous

The 5th International Conference on Life Science and Technology (ICoLiST)
AIP Conf. Proc. 3186, 020015-1–020015-7; https://doi.org/10.1063/5.0235708
Published under an exclusive license by AIP Publishing. 978-0-7354-5102-5/$30.00

in vitro studies, extracts of *A. sativum*, *C. mangga*, and *A. calamus* with a ratio of 36%: 36%: 28% could inhibit the growth of the fungus *Candida albicans* and showed high antioxidant activity [3].

The use of traditional herbal medicine has obstacles, that it takes a large dose to consume it. This is because in the body's digestive tract, plant extracts have low solubility so that they are stored low in blood plasma [4]. The way to overcome these obstacles is to make plant extracts in nanoparticles form. Nanoparticles are made as drug delivery agents capable of delivering drugs directly to target organs. Nanoparticles can maintain and control the active compounds in plant extracts during the delivery process so that side effects of the release of active compounds can be reduced [5]. This study aimed to determine the effect of nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* on the total cell leukocyte and total leukocyte types in mice that induced by cisplatin.

EXPERIMENTAL DETAILS

The experimental animals used were female mice (*Mus musculus*) with the Balb-C strain that weighed 20-25 grams and were 2-3 months old. This study used a completely randomized design (CRD) with 5 treatments and 6 replications as follows: Negative control (K-): Mice given distilled water; Positive control (K+): Mice induced by Cisplatin at a dose of 5 mL/kgBW and given nanoparticles extracts of *A. sativum*, *C. mangga*, and *A. calamus* at a dose of 0 mg/kgBW; Treatment 1 (P1) : mice induced by cisplatin and given nanoparticles of extracts of *A. sativum*, *C. mangga*, and *A. calamus* at a dose of 25 mg/kgBW; Treatment 2 (P2) : mice induced by cisplatin and given nanoparticles of extracts of *A. sativum*, *C. mangga*, and *A. calamus* at a dose of 25 mg/kgBW; Treatment 3 (P3): mice induced by cisplatin and given herbal fertility at a dose of 75 mg/kgBW. 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).

Mice were acclimatized for 1 week at room temperature (20-25^{o}C), fed BR1 pellets and drank ad libitum with mineral water. After 1 week, the mice were induced with Cisplatin at a dose of 5 mg/kgBW intraperitoneally using a single dose injection [6]. Then the estrus cycle was checked using the vaginal swab method twice a day [7]. Extraction of plant samples *A. sativum*, *C. mangga*, and *A. calamus* using the maceration method and then formed into nanoparticles [8]. The administration of extract nanoparticles used of gastric sonde method using a 2 mL syringe after being declared infertile with an irregular estrus cycle and in the diestrus phase. Extract nanoparticles were given every day for 15 days (three estrus cycles) as much as the dose of each treatment [9].

The blood sample of mice was taken from the tail as much as 1.5 mL. The total number of leukocytes was calculated using a hemocytometer and counting chamber. Mice blood was pipetted into the thoma leukocyte pipette to a limit of 0.5 and the Turk reagent to the 11 mark. Then the thoma leukocyte pipette was inverted. Discard as much as 2-3 drops of the solution in the pipette, then drop it into the counting chamber and wait for the cells to spread evenly. Then observed using a microscope at a magnification of 10x, and total leukocytes were counted in 4 large boxes in the counting room. After calculating the total leukocytes, then the total leukocyte per m formula is calculated, namely: cells counted × 20 (1:20) × 10 (0.1 mm): 4 (number of squares in m^2) [10]. The number of leukocyte types was calculated using the blood smear method. Dropped 1 drop of blood on the object glass and performed a blood smear. Then fixation was carried out using methanol and stained with 10% Giemsa. After staining, observations were made using a microscope at a magnification of 100×. Each type of leukocyte is calculated by multiplying the percentage of the number of each type found by the total number of leukocytes counted [10].

The leukocyte profile data were analyzed using SPSS 25 software. The data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity with the Levene's test. Normal and homogeneous data, continued with the one-way ANOVA test. If a p value <0.05 is obtained, there is a significant difference between treatments, so it needs to be continued to Duncan's further test [11]. The data is presented in the tables and diagram form.

RESULTS AND DISCUSSION

Nanoparticles Effect of *A. sativum, C. mangga*, and *A. calamus* to the Total Number of Mice Leukocyte that Induced by Cisplatin

The results of the One-Way ANOVA analysis showed that the data of total leukocyte had a significance value of $p = 0.000$ ($p < 0.05$) and showed that there is a significant difference between the treatment groups. The Duncan test showed that the P1 treatment was significantly different from all treatment groups. The treatments of P2 and P3 were significantly different from the positive control (K+) and not significantly different from the negative control (K-).

The average total number of leukocytes in each group based on Figure 1, was group K- (6766.67 ± 1592.06), group K+ (8133.33 ± 1809.60), P1 (3100 ± 629.29), P2 (5033.33 ± 1856.52), and P3 (5800 ± 2059.13). The highest leukocyte count was in the positive control group (K+) with an average of 8133.33 and the lowest leukocyte count was in the P1 group with an average of 3100.

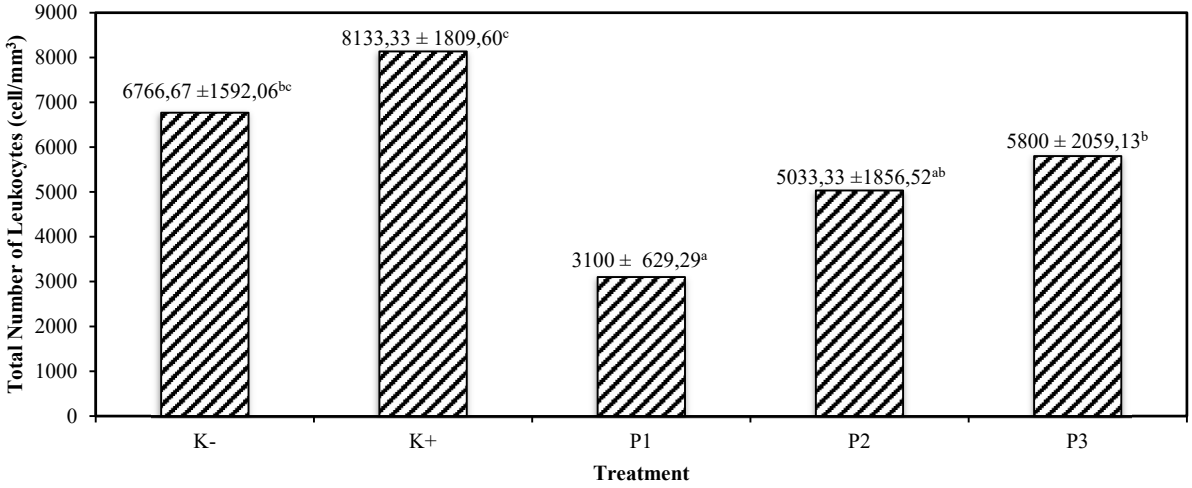

FIGURE 1. The average of total mice leukocyte. K - = distilled water, K + = cisplatin + nanoparticle extract 0 mg/kgBW, P1 = cisplatin + nanoparticle extract 25 mg/kgBW, P2 = cisplatin + nanoparticle extract 50 mg/kgBW, P3 = cisplatin + herbal fertility 75 mg/kgBW

Leukocyte observation was carried out to determine the health status of mice (*M. musculus*) in each treatment. The number of leukocytes in the blood can explain the ability of leukocytes to prevent the presence of pathogens, inflammation, or autoimmune diseases. An increase in the number of leukocytes indicates an increase in the immune system. While the number of leukocytes decreased and was still within normal limits, it indicated that there was no infection or inflammation in the body [12]. The results of this study showed that the total number of leukocytes in all groups was in the normal range. The normal total number of leukocytes in mice is about 3-14.2 x 103/µL of blood. The decrease in the number of leukocytes in the treatment group may occur because the extracts of *A. sativum, C. mangga*, and *A. calamus* contain flavonoids that are able to work as immunomodulators and immunosuppressants. Flavonoid compounds act as immunosuppressive factors of pro-inflammatory genes that can reduce the inflammatory response. Flavonoids can also inhibit the number of leukocytes in the area of inflammation by reducing the attachment of leukocytes to the endothelium so that it can reduce the inflammatory response [13].

Flavonoids have immunomodulatory and immunosuppressive activities that can increase the immune system and suppress the immune system when experiencing excess activity. When the function of the immune system decreases, flavonoids will send intracellular signals to cell receptors to make the function of the immune system increase. On the other hand, when the immune system is overworked, flavonoids will signal the immune system to reduce its activity [14]. Anti-inflammatory agents and immunosuppressants can induce lymphopenia and interfere with lymphocyte proliferation and differentiation. This will cause disruption of intracellular communication between leukocytes and the production of lymphokines (TNF, IL-1, and IL-2) so that it also interferes with macrophage activity [13].

Nanoparticles Effect of *A. sativum, C. mangga*, and *A. calamus* to the Total Type Number of Mice Leukocyte that Induced by Cisplatin

The data for calculating the average number of leukocytes in each treatment was shown in Table 1. Types of leukocyte data that were normally distributed and homogeneous were followed by the OneWay ANOVA test. The neutrophil and lymphocyte data had a significance value of $p<0.05$ indicating a significant difference between treatments and continued with Duncan's test. There was no significant difference between basophils and eosinophils, so further tests were not carried out. Monocyte data showed a significant difference and continued to the Games-Howell test.

Examination of the type of leukocyte was carried out to determine the exact cause of the imbalance in the immune system in the body. The five types of leukocytes have different immune functions. Lymphocyte levels are affected by medication, disease, and physical activity. Lymphocytes play a role in the body's immune response to fight bacterial and viral infections. Monocytes play a role in the second layer of defense that functions in phagocytosis. An increase in the number of monocytes indicates inflammation. Neutrophils play a role in fighting infection, inflammatory disorders, and tissue damage in non-infectious diseases. Eosinophils play an active role in the late stages of inflammation and have the ability to phagocytize. It also plays a role in parasitic infections and allergic reactions. An increased basophil count is associated with allergic reactions and granulocytic leukemia, whereas a decreased basophil count is associated with stress reactions, acute infections, and long-term steroids [15]. Each type of leukocyte was observed under a microscope at 1000x magnification as shown in Figure 2.

TABLE 1. The Average of Total Types Number of Mice Leukocytes

Treatment	The Average Percentage Count Types of Mice Leukocytes (%) ± SD				
	Neutrophil	Basophil	Eosinophil	Monocyte	Lymphocyte
K-	31.5 ± 4.67c	1.14 ± 0.51	3.33 ± 1.21	4.83 ± 0.75^{a}	59.83 ±4.99^{ab}
K+	21.67 ± 5.27b	2.57 ± 1.04	1.33 ± 1.30	12 ± 7.12^{abc}	63.67 ± 4.36^{ab}
P1	14.83 ± 3.25a	4.57 ± 3.01	3.33 ± 0.81	13 ± 4.42^{c}	66.33 ± 4.50^{b}
P2	13.83 ± 3.06a	1.42 ± 0.75	2.5 ± 2.25	6.33 ± 1.50^{a}	76.16 ± 5.30^{c}
P3	26.5 ± 6.15bc	0.57 ± 0.51	3.67 ± 3.26	12 ± 2^{b}	57.5 ± 8.91^{a}

Notes: Different notations showed a significant difference with a level of 5% ($p < 0.05$).

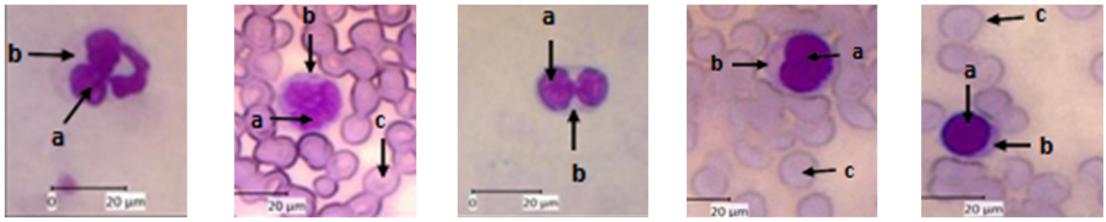

FIGURE 2. Morphology of Leukocyte Types Observed: (a) Neutrophil, (b) Basophil, (c) Eosinophil, (d) Monocyte, (e) Lymphocyte. Description: a. Cell nucleus, b. Cytoplasm, c. Erythrocytes

Neutrophil

The results of the One-Way ANOVA test analysis on neutrophils obtained a significance value of $p = 0.000$ ($p<0.05$) indicating that each treatment of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* to mice neutrophils that induced by cisplatin had significant differences. The average neutrophils obtained were still within normal values. The percentage of normal neutrophils in mice ranges from 10-40% [16]. Neutrophils that were still in the normal range indicated that the administration of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* did not cause inflammation or infection.

The increase and decrease in the number of neutrophils describes the body condition of the mice. Inflammatory mechanisms performed by neutrophils include chemotaxis, phagocytosis, release of reactive oxygen species (ROS) and granular proteins, as well as the production and release of cytokines. An increase in the number of neutrophils indicates inflammation or infection. An increase in the number of neutrophils in the blood is caused by acute stress, inflammation, and tissue damage or necrosis [17]. A decrease in the number of neutrophils can indicate that there is no inflammation or infection. When the body does not experience infection or inflammation, neutrophils will undergo spontaneous apoptosis for one to five days. On the other hand, when the body experiences an infection, a signal will appear that can prolong the life of neutrophil cells which then release inflammatory mediators [18].

Basophil

The results of the One-Way ANOVA test analysis on basophils obtained a significance value of p= 0.090 (p> 0.05), so that each treatment of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* to mice basophils that induced by cisplatin had no difference significant. The average basophils obtained were within normal limits. The percentage of normal basophils in mice ranges from 0.5-1.5% [19]. The number of basophils in the blood can describe the health of mice (*M. musculus*). The number of basophils that can be found in the bloodstream is relatively small. Basophils act as precursors of mast cells which have heparin (anticoagulant). Heparin will be released at the site of inflammation to prevent clotting and blood stasis [20]. An increase or decrease in the number of basophils beyond normal limits can be encountered in some circumstances. An increase in basophils is called basophilia. Basophilia can be found in conditions of granulocytic leukemia and basophilic myeloid metaplasia. While the number of basophils that are below normal is called basopenia. Basopenia can be seen in conditions of stress, acute infection, and long-term steroid therapy [21].

Eosinophil

The results of the One-Way ANOVA test analysis on eosinophils obtained a significance value of p=0.280 (p>0.05), so that each treatment of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* to mice eosinophils that induced by cisplatin had no difference significant. The average eosinophils obtained were within normal limits. The percentage of normal eosinophils in mice ranges from 0-4% [16]. Eosinophils that were in the normal range indicated that nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* did not cause allergies in mice. Eosinophils that were still in the normal range indicate that there was no parasitic infection and allergy in experimental animals [21].

An increase in the number of eosinophils in the blood indicates an infection in the body. When an infection occurs mainly due to parasites in the body, eosinophils will act by attaching themselves to a special surface on the molecule and releasing substances that can kill parasites, such as histamine. Eosinophils have the ability to attack parasites and have enzymes to neutralize inflammatory factors released by basophils or mast cells. Increased eosinophils in the blood circulation can be found when the body is attacked by parasitic and allergic diseases [22]. A decrease in the number of eosinophils that exceeds the normal range also indicates an immune response to infection. A decreased number of eosinophils is a form of immune response due to infection. A decrease in eosinophils below the normal value is called eosinopenia which occurs due to the migration of eosinophils to areas of inflammation [23].

Monocyte

The results of the One-Way ANOVA test analysis on monocytes obtained a significance value of p=0.013 (p<0.05) so that each treatment of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* to mice monocytes that induced by cisplatin had significant differences. The average monocyte obtained was above the normal value. The percentage of normal monocytes in mice ranges from 1-6% [16]. The number of monocytes in the blood could determine the condition of mice experiencing infection from foreign substances or not. Monocytes could be in the form of macrophages that can phagocytose bacteria and other microorganisms. Monocytes carry out an immune response by producing proteins from a complement and produce substances that affect the chronic inflammatory process [24].

The flavonoid and phenolic compounds contained in *A. sativum*, *C. mangga*, and *A. calamus* can stimulate macrophage phagocytosis. Flavonoid compounds play a role in the inflammatory process, lymphocyte production, granulocyte production, and cytokine production based on the mechanism of protection against free radicals [23]. Flavonoids can also increase the phagocytic activity of macrophages and the proliferation of lymphocyte cells in the body. An increase in the number of monocytes as a homeostatic response in the body. This is because many monocytes turn into macrophages and migrate to tissues in response to chemotactic stimuli to deal with inflammation [25]. So that the increase in monocytes in mice treated with nanoparticles *A. sativum*, *C. mangga*, and *A. calamus* is a form of the body's immune response to the presence of free radicals due to cisplatin induction [26].

Lymphocyte

The results of the One-Way ANOVA test analysis on lymphocytes obtained a significance value of p=0.000 (p<0.05) so that each treatment of nanoparticle extracts *A. sativum*, *C. mangga*, and *A. calamus* to mice monocytes that induced by cisplatin had significant differences. The average lymphocyte obtained was still within normal limits.

The percentage of normal lymphocytes in mice ranges from 55-95% [16]. The number of lymphocytes in the blood can indicate the mice are inflamed or not. Lymphocytes are part of the adaptive immune response. Lymphocytes are derived cells from lymphoid progenitor cells that can help mediate specific immune reactions against pathogens and can recognize or remember these pathogens in the next attack. Lymphocytes also function to respond to foreign antigen attacks by producing antibodies in the blood circulation [27]. There are two kinds of lymphocytes found in the body, namely B lymphocytes and T lymphocytes. T lymphocytes function in the cellular immune response, in the form of T helper cells that assist the activity of cytotoxic T cells to kill and attack intracellular foreign antigens. While B lymphocytes function to produce antibodies that function in the humoral immune response, namely by attacking intracellular pathogenic microorganisms [28].

An increase in the number of lymphocytes indicates the presence of foreign antigens that attack the body. The number of lymphocytes increases due to the presence of pathogens such as viruses, bacteria, and parasites that attack the body and cause lymphocytes to respond by producing antibodies. A high number of lymphocytes occurs as a physiological response of the body to carry out destruction of tissue cells or organs that undergo apoptosis [21]. While the decrease in the number of lymphocytes is due to the migration of lymphocyte cells from the blood circulation to the body tissues [29].

SUMMARY

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 and the total type number of leukocytes. In the percentage of neutrophils, monocytes, and lymphocytes the treatment was able to maintain their numbers in the normal range. Meanwhile, in the percentage of basophils and eosinophils, the treatmentwas able to increase its number in all dosage range.

ACKNOWLEDGMENTS

This research was supported by Department of Biology, Faculty of Science and Technology, The State Islamic University of Maulana Malik Ibrahim Malang. The authors thank all parties involved in this research.

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