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The Effect of Combined Administration of Sea Urchin Gonad and Aloe Vera Suspension on the Enhancement of Macrophage Phagocytosis Activity in Male White Rats Induced by Salmonella Typhimurium

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KEYWORDS
Aloe Vera, Macrophage Phagocytes Activities, Tripneustes Gratilla.

ABSTRACT
This research determined the influence of the administration of combined gonadal sea urchin suspension and aloe vera to improve the macrophage phagocyte activities of male mice (Rattus norvergicus). This experimental laboratory research used a posttest-only control group design with white mice (Rattus norvegicus). The researcher grouped the mice into three groups: the negative control group with CMC-Na (0,5% /kg Weight); the positive group with Levamisole 0,9 mg/ 200 weight; and the intervention group with combined gonadal sea urchin suspension, Tripeneustes gratila, with a dose of 2,2700g/kg Weight, and the extracted aloe vera for 15ml. The researcher provided the treatment for 12 days, orally, twice a day with the induction of Salmonella typhimurium of 104 CFU 0,1 ml on the fifth day. The outcome of the macrophage phagocyte capability on the thirteenth day was calculated with the phagocytes index. The One-Way Anova test of gonadal sea urchin suspension, Tripneustes gratila with a dose of 2.2700 g/kg Weight with the extracted aloe vera indicated the highest phagocytes index, 2.28 ($p<0.05$) than the control group. The combined gonadal sea urchin suspension, Tripneustes gratilla, with the extracted aloe vera could improve the macrophage phagocyte activities.

Introduction

Salmonella typhi (26.2%) is a pathogenic microorganism often isolated in the Asian region (Wiedosari, 2020). Typhoid fever is an acute infection in the small intestine caused by Salmonella typhi contamination (paratyphi). This infection leads to serious complications, such as ileum perforation, bacteremia, and endovascular infections occurring in up to 10% of cases (dr.Subangkir, 2016), especially in individuals who

experience typhoid symptoms for about fourteen days without receiving adequate treatment. Typhoid fever is an epidemic disease that affects public health, with a Case Fatality Rate (CFR) of 1-4%, and in cases without treatment, CFR can increase to 20%, particularly in Indonesia.

Salmonella typhimurium (Enterobacteriaceae Family) is a Gram-negative bacterium with flagella, facultative anaerobic, encapsulated, and non-sporulating, appearing pink in Gram staining (Gram-negative). It is a single bacillus bacterium that causes typhoid, with dimensions of 2 μ to 4 μ × 0.6 μ, possessing flagella (except S. gallinarum and S. pullorum) (Hanum, 2016), and it does not form spores. Therefore, the cellular immune system plays a crucial role (Sangadah & Kartawidjaja, 2020). This intracellular microscopic organism activates macrophages to produce IL-12, which initiates NK cells and converts Th0 cells into Th1 cells, subsequently secreting IFN-γ to enhance microbe killing and lysis of infected cells by CD8+ cells. Salmonella typhimurium resides in the gastrointestinal system (small digestive tract) of humans and animals. The ideal temperature for the development of Salmonella typhimurium is 37°C, and it thrives at a pH of 6-8 (Supiati, 2022).

One of the new fields in pharmacology under investigation is immunomodulators (Sofiakmi et al., 2016), particularly the development of substances that enhance immune responses. Immunomodulators are substances that can restore the balance of the immune system, mainly used in chronic infectious diseases and cancer cell growth. In the field of pharmacy, immunomodulators are derived from both biological and non-biological sources and play a role in enhancing immunity against diseases, especially infectious diseases. The combination of molecules and tissues involved in resistance to infection is referred to as the immune system. The coordinated response of cells, particles, organisms, and different substances against different organisms and substances is known as the immune system (Sangadah & Kartawidjaja, 2020).

The morphological characteristics of sea urchins are round in shape and covered with stiff hairs or spines. Sea urchins (Tripneustes gratilla) have a body height of 4.3-5.4 cm and a flattened round body shape, with a body diameter of 4-7 cm (Sapianus, 2020). They have a dark green body color with reddish-black interlacing on their ambulacral regions and are equipped with spines all over their body surface (Tupan *et al*., 2017).

Sea urchins, specifically their gonads, contain 47.79% protein (Wang et al., 2018). The high protein content in sea urchin gonads can act as an immunomodulator. The amino acids and proteins present in sea urchins are effective in regenerating cells (Kakilo, 2020). Carotenoids, such as β-carotene, α-carotene, and γ-carotene, are present in sea urchins and act as precursors to vitamin A, lipid oxidation inhibitors. They can increase the levels of IL-2, which, in turn, enhances the activity of NK cells involved in the immune response. Among the 600 types of carotenoids in nature, only 10% exhibit provitamin A activity (Girsang, 2020). Vitamin A deficiency affects humoral immunity, where cell-mediated immunity is impaired. The production and maturation of lymphocytes decrease with a lack of vitamin A. Studies in Indonesia have found that the CD4+/CD8+ T-cell ratio is low in peripheral blood lymphocytes in children with xerophthalmia compared to non-xerophthalmic controls (Dettleff *et al.*, 2020). After vitamin A supplementation, the proportion of CD4+ to CD8+ T cells and the percentage of CD4+ T lymphocytes increase (Kakilo, 2020).

Calcium plays a role in increasing cell immunity, as calcium ions enter mast cells, which contain granules that release when the cells are stimulated by the binding of immunoglobulin E antibodies (dr. Subangkir, 2016). Fatty acids, specifically a deficiency

of linoleic acid, can suppress antibody responses, and high consumption of fatty acids can reduce T-helper cells and cytokine production (Biopharmaceuticals, 2013).

Aloe vera (L.) Webb, known as Aloe vera, is a cactus plant that contains 99-99.5% water with an average pH of 4.5. It contains more than 75 different components, including vitamins, minerals, enzymes, sugars, lignin, saponins, anthraquinones, sterols, amino acids, and salicylic acid. It contains vitamins such as C, A, E, B-1, 2, 6, 12. Vitamin A deficiency results in a slight reduction in thymus gland weight, decreased lymphocyte proliferation response to mitogens, decreased specific antibody production, decreased in vitro T lymphocyte proliferation, and increased bacterial adherence to respiratory epithelial cells (Liu *et al.*, 2019). Vitamin C can increase the expression of cytosolic phospholipase A2 and cyclooxygenase (COX)-1, triggering the release of cytokines and lymphocytes in humans, enhancing macrophage phagocytosis (Jain *et al.*, 2016).

Contains digestive enzymes including amylase, lipase, lactase, and other enzymes, which are easily damaged by heat (Jain *et al.*, 2016). Contains Sodium, Potassium, Calcium, Magnesium, Zinc, Chromium, Selenium, and Iron (Rahman et al., 2017). Carbohydrates are derived from the mucilage layer beneath the plant's epidermis, comprising 25% solid fractions, consisting of monosaccharides. A long-chain polysaccharide consisting of glucose and mannose known as glucomannan (Beta-(1,4)-linked to acetylated mannan) acts as an immunomodulator (Gao *et al.*, 2019). Anthraquinone contains 12 phenolic compounds found in the latex of the Aloe vera (L.) Webb plant. It is bitter and consists of free anthraquinone and its derivatives: (babbaloinIO-(1141-anhydroglucose)-aloe-emodin-9-anthrone), isobarbaloin, anthrone-glycosides, and chromones. In large amounts, it has a purgative effect, while at lower concentrations, it has the potential to act as an antimicrobial and analgesic ((Gao *et al.*, 2019). Saponin constitutes three percent of the gel and functions as a cleansing, antiseptic, and antimicrobial agent against viruses, bacteria, fungi, and yeast (Rahman et al., 2017).

Fat consists of cholesterol, campesterol, sitosterol, and lupeol (Jain et al., 2016). Salicylic acid acts as an anti-inflammatory and antibacterial agent and has a kerolytic effect that aids in the healing of necrotic tissue debris (Liu et al., 2019). Amino acids contain 20 essential amino acids required by the body, with seven of these amino acids being non-synthesizable by the body. Sterols include campesterol, sitosterol, and lupeol. Aloe vera (L.) Webb, which contains acemannan, can enhance monocyte function, macrophage activity, cytotoxicity, stimulate T cells, and boost or increase the activity of candidacidal macrophages (in vitro). Furthermore, acemannan enhances and stimulates macrophages to release interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) (Jain et al., 2016).

Alternative therapy with natural immunomodulating substances (Mahmoud *et al.*, 2022) is aimed at reducing the colony count of Salmonella typhimurium in sea urchin gonads (Tripneustes gratilla) and Aloe vera gel (Aloe vera (L.), Webb). Sea urchin gonads (Tripneustes gratilla) have a place in the Echinodermata class, which is not commonly known to the general public. Sea urchin gonads contain 47.79% protein (Inguglia *et al.*, 2020). The high protein content in sea urchin gonads can be used as an immunomodulator (Fitria Nurma Putri, 2012). In developed countries that have adopted a diet-focused approach, sea urchins are a food source with high dietary benefits, containing amino acids and unsaturated fats that can reduce cholesterol levels in the human body (Sastika *et al.*, 2014).

Aloe vera gel contains acemannan, which has the activity of enhancing monocyte function, macrophage movement, cytotoxicity, strengthening the immune system against microorganisms, and enhancing candidacidal macrophage activity (in vitro). Moreover, acemannan stimulates and activates macrophages to produce interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) (Rich *et al., 2012)*. Amino acids help in the repair of new cells by expanding lymphocyte expansion, and the enzymes contained in Aloe vera gel help eliminate dead cells from the epidermis.

Given these conditions, sea urchin gonads (Tripneustes gratilla) and Aloe vera gel (Aloe vera (L.) Webb) can be utilized as immunomodulators. However, there is still limited research to prove this. Based on these reasons, it is necessary to demonstrate the phagocytosis activity of macrophages in male white rats infected with Salmonella typhimurium using sea urchin gonad powder (Tripneustes gratilla) and Aloe vera gel (Aloe vera (L.) Webb).

Research Methods

This research is experimental. The object used in this study was the phagocytosis activity of macrophage cells of male white rats Wistar strain infected with Salmonella typhimurium. The variables used in this study were independent variables, namely the dose of sea urchin gonad powder of 2.2700 g / kg body weight and aloe vera juice of 15 ml. The dependent variable is the phagocytosis activity of macrophage cells in white mice infected with Salmonella typhimurium. Controlled variables were age, strain, body weight of male white rats, rat maintenance conditions (cage, food, maintenance process), Salmonella typhimurium concentration 104 CFU / ml 0.1 ml.

The materials used are white rats (Rattus norvegicus) male Wistar strains, age approximately 2-3 months, body weight 175-200 grams, animal feed BR2, CMC-Na, , sea urchin gonad powder, aloe vera juice, Salmonella typhimurium bacteria 104 CFU / ml, aquadest, 70% technical alcohol, Merck ® technical giremsa, Gibco Phosphate Buffer Solution (PBS), RPMI-1640 Sigma ® ®, Latex Beads Sigma Chem. Co. The tool used is a centrifuge Heraeus Laborage 400R, Signora juicer, hemocytometer, NucN® plate, NucN® coverslips, incubator, slip cover, Envirco laminar air flow, and Nikon SE microscope.

The following is a grouping of test animals:

- Negative Control Control: Group given 0.5% CMC-Na for 12 days orally, twice daily
- Positive Control Group: The group with male white rat tails received standard feed, orally administered Levamisol (0.9 mg/200 g mice), every morning and evening for 12 days
- Treatment Group: Group with a dose of sea urchin gonad powder 2.2700 g / kg body weight rats and combined with aloe vera juice 15 ml, twice a day.

Negative controls, positive group and treatment group, infected with Salmonella typhimurium 104 CFU / ml 0.1 ml on day 5, then on day 13 all test animals were killed, intraperitoneal fluid was taken, after being prepared then observed under a light microscope with magnification 40x10 and calculated the phagocytosy index.

Macrophage Isolation

The rats were killed, then placed in the supine position, skinned part of the abdomen, and cleaned from the peritoneal sheath with 70% alcohol (v / v) and injected

±15 ml of cold RPMI into the peritoneal cavity. Then let stand for ± 3 minutes while gently shaking (so that macrophages attached to the peritoneal cavity and around the intestine can be released and suspended in RPMI medium) Peritoneal fluid is removed from the peritoneal cavity (selected in the non-fatty part and away from the intestine), then the fluid is inserted in the volken tube. Intraperitoneal fluid is centrifudated 2500 rpm for 10 minutes. The formed supernatant is removed and then added up to 8 ml of "complete RPMI medium" (containing 10% PBS (v/v)). The number of cells is calculated with a hemocytometer, then resusted with a complete medium. The calculated cell suspensions were grown in plates of 21 wells that had been given round coverslips, each well containing 5x105 macrophage cells. Cells are incubated in

Incubator CO2 5% 370C for 30 minutes, then added complete medium 1 ml per well and incubated continued for 2 hours. Cells are washed with RPMI twice then added complete medium 1 ml per well and continued incubation for up to 24 hours.

Phagocytosis of macrophages with latex beads

The ability of non-specific phagocytosis in vitro using latex beads diameter 3μm. Latex is resuspensioned in PBS so that a concentration of 2.5x107/ml is obtained. Peritoneal macrophages cultured the day before were washed twice with RPMI, added latex suspension 5x105 cells per well and incubated in a 5% 370C CO2 incubator for 60 min. The cells were then washed with PBS three times to remove the unphagocytosed latex. After that it is dried at room temperature and fixed with methanol for 30 seconds. Next, the methanol is removed and the coverslips are allowed to dry. After drying, the coverslips are stained with Giemsa 20% (v/v) for 30 minutes. Washed with aquadest, lifted from the culture well and dried at room temperature, then coverslips attached to glass objects (Istini, I., &; Santoso, B. (2018). One hundred macrophage cells were observed and counted the number of macrophages phagocytosed by latex particles and the number of latex phagocytosed by macrophages. Observation (phagocytosis index) of macrophages is carried out using a light microscope with a magnification of 40x10 with the following formula:

$$\frac{\text{jumlah makrofag makan lateks}}{100} \times 100\% \times \frac{\text{jumlah lateks yang dimakan makrofag}}{\text{jumlah makrofag makan lateks}}$$

Data analysis

Phagocytosis activity data obtained from each treatment group calculated phagocytosis index values. Furthermore, the phagocytosis index value of each treatment group was carried out statistical tests using SPSS for Windows version 26.

Results and Discussions

From the results obtained showed 83.33% that the drying loss value in Tripneusted gratilla and wet showed a difference, where the drying loss value of Tripneusted gratilla dry was smaller than that of Tripneusted gratilla wet. This is because the high water content in wet tripneusted gratilla causes more compounds to be lost than dry tripneusted gratilla. The value of the drying shrinkage parameter obtained indicates the magnitude of the compound lost in the drying process. Based on the test results that have been obtained and have been presented in the form of tables and figures, it is

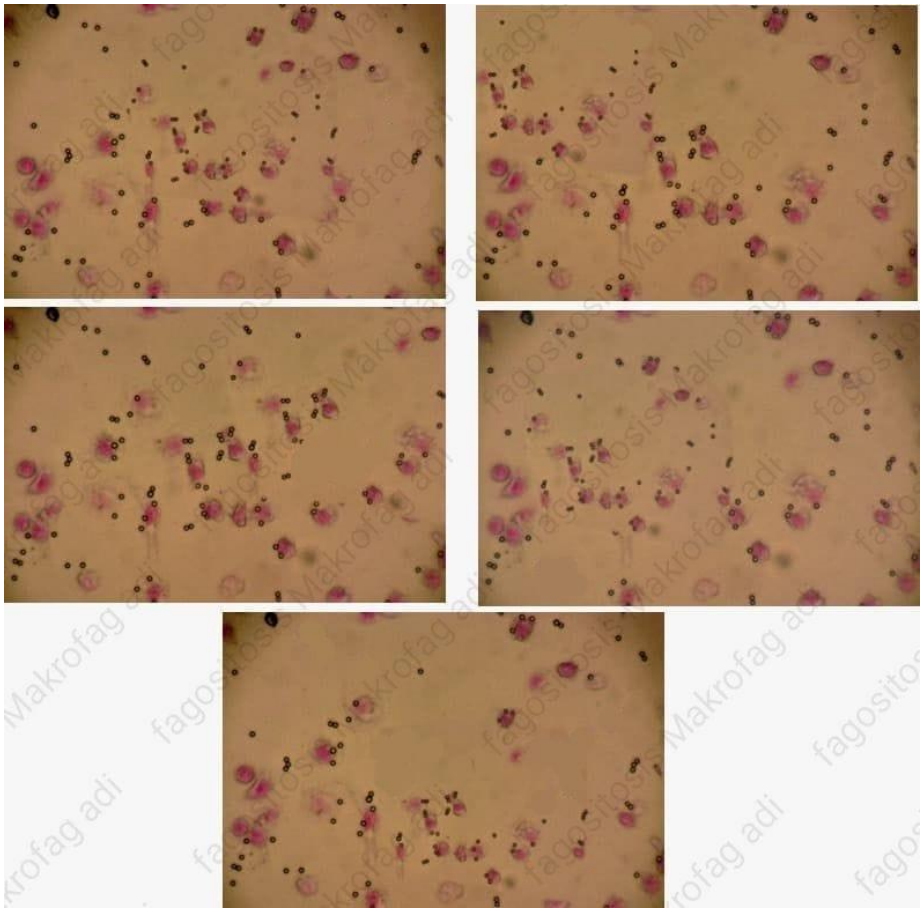
supported by the main components of the non-specific immune system are physical and chemical defenses such as epithelium and antimicrobial substances produced on the surface of the epithelium, various types of proteins in the blood including components of the complement system, other inflammatory mediators and various cytokines, phagocytic cells namely polymorphonuclear cells and macrophages and cells Natular killer (NK). One of the body's efforts to defend itself from the entry of antigens, such as bacterial antigens, is to destroy the bacteria in a nonspecific manner by the process of phagocytosis, regardless of the small differences that exist between foreign substances (dr. Subangkir, 2016).

Salmonella typhimurium was used as a research antigen. Bacteria infect experimental animals intraperitoneally, and macrophages already present in the peritoneum immediately phagocytosis foreign bacteria due to the presence of receptors for phospholipids (Biopharmaceuticals, 2013), while the function as effector cells that destroy microorganisms as well as malignant cells and foreign objects is possible because these cells have a number of lysosomes in the cytoplasm containing hydrolases and peroxidases which are destructive enzymes (Droz et al al., 2019). All mice were necropsy on the 13th day, intraperitoneal fluid was taken to observe phagocytosis of macrophage cells, and the phagocytosis index was calculated, as table II.1 Average value from the calculation results, and photo images of Phagocytosis of Macrophages of each treatment

Table.1 Average value of the calculation results

Group	Latex-eating macrophages	Latex eaten by macrophages	Phagocytosis Index
a. Control Negative	68	127	1,27
	61	160	1,6
	64	115	1,15
	61	117	1,17
	75	163	1,63
	71	136	1,36
$\bar{x}$	66,67	136,33	1,36
	95	224	2,24
b. Positive Groups	85	241	2,41
(Levamisol orally	98	220	2,2
(0.9 mg/200 g rats))	99	217	2,17
	100	276	2,76
	90	162	1,62
$\bar{x}$			
c. Treatment Group	94,5	223,33	2,23
(Dose of sea urchin	97	226	2,26
gonadal powder	87	243	2,43
2,700 g/kg body	100	222	2,22
weight 2 times daily)	101	219	2,19
	102	278	2,78
$\bar{x}$	92	164	1,64

	96,5	225,33	2,28



Microscopic image of Replication of 6 test animals

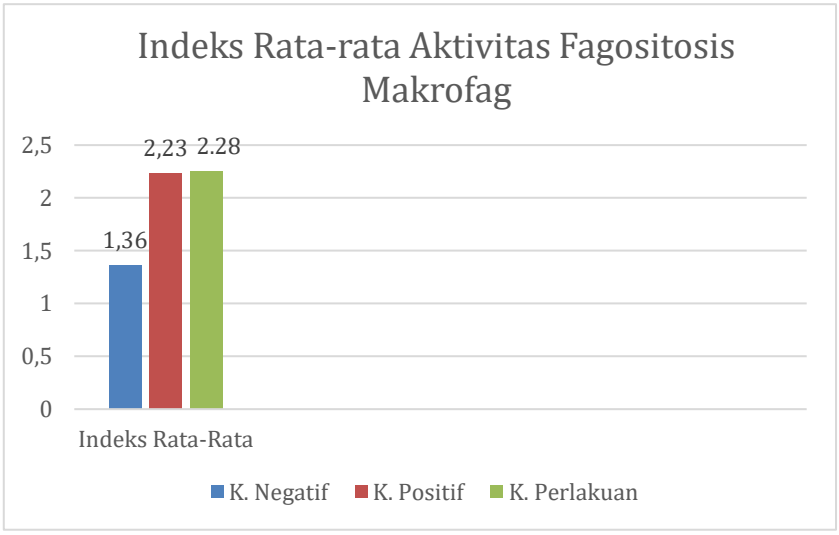


Figure 1. Phagocytosis Activity Diagram of Macrophage Cells

The increase in macrophage phagocytosis activity can be observed in Figure 1. In the treatment group, the highest phagocytosis activity was found in the dose of 2,2700 g/kg BW of sea urchin gonad powder and Aloe vera gel, followed by the phagocytosis activity value in the positive group, which received a dose of Levamisole 0.9 mg/200 BW, and the negative control group.

To confirm the significance of the difference in the mean values of macrophage phagocytosis activity among the three groups, a statistical analysis was conducted using assumption tests to determine whether parametric or non-parametric analysis would be used. Assumption tests include testing the normality of the data and homogeneity of variances, the results of which can be seen in the following table.

Table 2. Normality Test Results

Group	*p-value*	Information
Negative Control	0,243	Normal
Positive Groups	0,632	Normal
Treatment Group	0,632	Normal

The table above shows that each group has a normally distributed value of macrophage phagocytosis activity. The next assumption test is the variant homogeneity test, the results of which can be seen in the table below:

Table 3. Variant homogeneity test results

Levene Statistic	df1	df2	Sig.
0,221	2	15	0,804

According to the table above, the Levene test with a significance level of 0.804 (>0.05), there was a uniform homogeneous variation in data on the value of macrophage phagocytosis activity in the three groups.

The one-way ANOVA assay is used to confirm the significance of macrophage phagocytosis activity between groups, since the parametric assay assumption of the mean difference of two or more groups has been satisfied. The results of the One Way ANOVA test are shown in the table below:

Table 4. One Way Anova Test Results

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.236	2	1.118	13.704	.000
Within Groups	1.224	15	.082		
Total	3.459	17			

The table above shows that there is a significant difference in the value of macrophage phagocytosis activity between the three groups, as seen from the acquisition of a significant level of 0.000 (<0.05). Next, to find out the location of the differences between groups, a post hoc test was carried out whose results can be seen in the table

below:

Table 5. Post Hoc Test Result

(I) Kelompok	(J) Kelompok	Mean Difference (I-J)	Sig.
Negative Control	Positive Groups	-0,870(*)	0,000
Negative Control	Treatment Group	-0,495(*)	0,027
Treatment Group	Positive Groups	-0,375(*)	0,49

Shows results comparing the level of phagocytosis activity of macrophages between the two groups. The mean value of macrophage phagocytosis activity between the negative control group and the treatment group, i.e. the dose of sea urchin gonadal powder (Tripneustes gratilla) was significantly shown at p = 0.027 (<0.05). The average phagocytosis activity of macrophages in the treatment group was higher than the average phagocytosis activity of macrophages in the negative control group. This illustrates that doses of sea urchin gonadal powder (Tripneustes gratilla) can increase the phagocytosis activity of macrophage cells in male wistar white rats infected with Salmonella typhimurium.

The difference in the average value of macrophage phagocytosis activity, when viewed from among the negative control group, which was given orally CMC Na 0.5% 5 ml with the treatment group, namely the combination of doses of sea urchin gonad powder (Tripneustes gratilla) with aloe vera juice was also significantly indicated by p = 0.49 (< 0.05). The average value of phagocytosis activity of macrophages of the negative control group was lower than the average value of phagocytosis activity of macrophages of the treatment group. This showed that the dose of sea urchin gonad powder (Tripneustes gratilla) 2.2700 g / kg body weight and aloe vera juice had an effect on increasing macrophage cell activity in male white rats. As for the shortcomings of this study, researchers realized after that everyone can be a jury but not everyone is self-aware, the research process has shortcomings in bureaucracy for a long time in reading research results, tools and laboratory materials STIKES Telogorejo that do not yet exist, the search for the main research material that is difficult to support the erratic transition weather.

Conclusion

Based on this study, it can be concluded that there is an increase in phagocytosis macrophage activity in male white rats Wistar strain between those given sea urchin gonad powder (Tripneustes gratilla) with aloe vera juice (Aloe vera (L.) Webb) with administration of sea urchin gonad powder (Tripneustes gratilla).

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