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The Effect of Standarized Extract Pegagan Embun (*Hydrocotyle sibthorpioides* Lam.) on Total Leukocyte and Percentage Leukocytes in Male White Mice Exposed to H5N1 Virus Antigen

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Abstract:- **Pegagan embun has been used to increase endurance. In this study, the effects of Pegagan embun extract on the number and percentage of leukocyte cells exposed to the H5N1 antigen were observed. As many as 35 experimental animals were used, divided into 7 groups, by varying the time of administration of the H5N1 antigen. Groups 1 and 2, given the test extract for 7 days and induced H5N1 antigen on days 1 and 7 were evaluated on day 8. Group 3 was given a test extract for 3 days then induced antigen on day 4 and continued with giving the test extract until day 7. In groups 5 and 7, only H5N1 antigen induction was given on day 1 and evaluated on day 8 and day 5. Groups 4 and 6 were only given the test extract for 7 and 4 days. Observations were made on the total number and percentage of leukocyte cells. After being counted, the total leukocyte cells for groups 1 to 7 were 8.54 respectively; 10.06; 12.08; 7.52; 8, 26; 5.62; and 6.42 x 103 µL, meanwhile for the presntase of leukocyte cell types the yield of eucinophils was 2.20%; 5%; 5.60%; 4.80%; 3%; 2.40% and 5.40%, stem neutrophils 23.8%; 21.4%; 28.20%; 15.40%; 29.80%; 29.20% and 23.80%, segment neutrophils 16.8%; 17.8%; 14%; 28.2%; 14.8%; 23.2% and 14.6%, 17.6% monocytes; 18.2%; 15.4%; 11.2%; 13.2; 10.4% and 17.8%. From the results of statistical analysis it can be concluded that giving Pegagan embun extract to male white mice exposed to the H5N1 antigen can increase the number of leukocytes and the percentage of leucocyte cell types that increase are neutrophil cells, eucinophils, and monocyte cells.**

Keywords:- *Hydrocotyle sibthorpioides Lam.; Immunostimulan; Leukocyte percentage; Total leukocyte.*

I. INTRODUCTION

Immune-mediated disease is a significant problem in developing countries. An environment that is rich in various types of pathogenic microorganisms, such as viruses, bacteria, fungi, protozoa and parasites is the main factor causing infection in humans [1].

In the current COVID-19 situation, the ability of the immune system to maintain individual health is being tested with a formidable challenge. It can be said that the immune system is the main force whose role is very important where the success of its work will greatly affect the occurrence or absence of infectious diseases including COVID-19 in a person. In a state of being infected with COVID-19, the immune system plays a large role in the success or failure of the treatment process, this is supported by data that most of the Covid-19 patients who are not helped are those who are elderly and have comorbidities that aggravate their condition. Significant increases in the number of neutrophils, leukocytes, and neutrophil-lymphocyte ratio were reported to be more common in severe cases of COVID-19 than mild cases [2].

One of the popular traditional medicines in China that is often used is pegagan embun (*Hydrocotyle sibthorpioides* Lam.) [3]. which has properties such as eliminating swelling (anti-swelling), anti-inflammatory, laxative urine, antibiotics, fever reducers, neutralize toxins (detoxificans).), and phlegm laxative (expectorant) [4]. Research conducted by Farong Yu et al reported that H. sibthorpioides extract produced excellent antitumor effects and showed the ability to affect the immunological function of mice [5].

Previous research that has been carried out and published is the immunostimulant activity of pegagan embun in white male mice, which resulted in a significant increase in the activity and capacity of macrophages and the total number of leukocytes, and a significant increase in the percentage of lymphocytes while neutrophil cells decreased significantly [6], then the anti-inflammatory activity of the extract of pegagan embun which was administered topically which at a concentration of 0.5; 1 and 2% can have an anti-inflammatory effect [6].

Based on the description above, researchers are interested in conducting research on determining the effect of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) on the total number of leukocytes and the percentage of leukocyte cell types in male white mice by varying the timing of antigen administration in influencing the improvement of the body's defense system.

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II. MATERIAL AND METHODS

A. Equipment

The tools used are Evaporator (Buchi R-210 Rotavapor), UV-vis spectrophotometer (Thermo Scientific Genesys 10S UV-Vis), UV-lamp (Camag), beaker glass (Pyrex), Silica gel 60 F_{254} (Merck), sentrifuge (Gemmy PLC-03), oven (Memmert), haemocytometer (Neubauer), spektrofotometer (BIO-RAD), object glass (Slides) mikroskop optilab (Motic), elisa reader (BIO-RAD), incubator (Biosan).

B. Materials

Ethanol P (Merck), Chloroform P (Merck), Methanol P (Merck), glacial acetic acid P (Merck), rutin, silica gel 60 F254 (Sentana), KI (Merck), FeCl3 (Merck), HgCl2 (Merck), AlCl3 (Merck), Mouse NK cell kit and CD8 cells (Bioassay Technology Laboratory), H5N1 Vaccine no. batch VF09B30 (Caprivac® AI-K), Geimsa dye (Merck) and Turk solution (Segara husada).

C. Procedure

1) Preparation of sample

The sample used for this study was pegagan embun taken in Batu Gadang Village, Lubuk Kilangan District, West Sumatra, Padang City. The identification of pegagan embun was carried out at the ANDA Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA) Andalas University (UNAND) Padang, West Sumatra.

2) Preparation of extract

A total of 1 kg of pegagan embun that has been dried and has been finely ground, macerated using 70% ethanol solvent. Put one part of dry simplicia powder into the macerator, add 10 parts of the solvent. Soak for the first six hours while stirring occasionally, then let stand for 18 hours and strain. Repeat the filtering process twice with the same type and amount. Collect all the maserate, then evaporate with a rotary evaporator until a thick extract is obtained.

3) Extract standardization

Non-specific and specific characterization of pegagan embun extract was carried out, non-specific characterization was drying shrinkage, total ash content and acid insoluble ash content, while specific characterization was organoleptic test, identity parameter, chemical content test of extract, thin layer chromatography and determination of total flavonoid content.

4) Dosage planning and induction

Using 1 variant, the optimal dose of Pegagan embun extract is 200 mg/kgbb (Aldi et al., 2020). The induction used is the H5N1 Cavrivac® AI-K vaccine with a dose of 50µL administered intramuscularly.

5) Treatment of experimental animals

Healthy mice (Mus musculur strain BALB/c) with a body weight of 20-25 g, totaling 35 individuals and randomly divided into each group consisting of 5 individuals. The treatment was given once a day in the afternoon, for (group I) the pegagan embun extract was vaccinated for 7 7 days and on the 8th day was evaluated (group II) mice were vaccinated on the first day then after that they were given the extract for 7 days. day, (group III) the extract was given 3 days before vaccination then on day 4 the mice were vaccinated 3 days after the extract was given, (group IV) consisted of mice that only received the Avian influenza vaccine on day 1 and Na. CMC for 6 days, not treated with pegagan embun extract, (group V) each mouse received got pegagan embun extract 200 mg/KgBW for 7 days without being vaccinated, (group VI) only received the vaccine during day 1 and Na. CMC for 3 days, then (group VII) only received the extract for 4 consecutive days, before being treated the experimental animals were acclimatized for 7 days. The treatment process lasted for 7 days and on the 8th morning an evaluation was carried out [7].

6) Calculation of Total Leukocyte Cell Count

After being given the extract for 7 days, the mice's blood was taken from the tail vein using a leukocyte pipette to the "0.5" mark then added turk's solution to the "11" mark. Then shake it for some time, then discard the first 1-2 drops then drop it into the haemocytometer counting room and then examine it under a microscope with a weak magnification. The formula for the number of leukocytes per m is: cells counted x 20 (1:20) x 10 (0.1 mm): 4 (number of squares in m²) or number of cells counted in squares multiplied by 50 [8].

$$\text{Jumlah total leukosit} = jumlah\ sel\ x\ \frac{20}{40}$$

7) Calculation of Leukocyte Percentage

After 7 days of administration of the extract, a blood smear was made to calculate the percentage of leukocytes by taking the blood of mice from the tail vein, then putting blood on a slide and using another slide to flatten the smear, wait until it dries. After drying, it was dripped with methanol to coat the entire smear (5 minutes). Drops of 10% Giemsa solution then let stand for 20 minutes. Wash with distilled water, put a drop of immersion oil and observe under a microscope [8].

8) Data analysis

The data obtained were analyzed statistically with the one-way analysis of variation (ANOVA) method followed by Duncan's test [6].

III. DISCUSSION

Plant identification was carried out at the Laboratory Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University (ANDA) Limau Manih Campus, Padang, West Sumatra. Based on the results of the identification of the plant used in this study is pegagan embun (*Hydrocotyle sibthorpioides* Lam.) Apiaceae family.

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The process of making pegagan embun extract begins with the collection of fresh pegagan embun plants and then cleaned of impurities, washed with running water and then air-dried to become dry simplicia, then after the simplicia is dried it is mashed using a blender and powdered simplicia is obtained. Sample maceration was carried out using 70% ethanol solvent [10]. From 1037.05 grams of simplicia powder, 170.74 grams of thick extract were obtained and the extract yield value was 16.46%, then standardization of pegagan embun extract was carried out, the drying shrinkage of the extract obtained was 3.95%, this meets the provisions of the Indonesian Herbal Pharmacopoeia where The drying shrinkage of the pegagan embun herb extract was not more than 10% and the result of determining the total ash content of the pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) was 2.55%, this fulfilled the provisions of the Indonesian Herbal Pharmacopoeia where the total ash content of the thick extract of the pegagan embun herb was not more than 16, 6% while the acid insoluble ash content produced is 0.07% according to the Indonesian Herbal Pharmacopoeia, the insoluble ash content of the thick extract of pegagan embun herb is not more than 2.3%.

The organoleptic examination was aimed at a simple initial introduction of the pegagan embun extract used, namely in the form of a thick extract having a characteristic dark brown color and bitter taste. The chemical content test of the extract included a phytochemical test, which showed that the pegagan embun extract was positive for flavonoids, phenolics, terpenoids, and saponins.

In the Thin Layer Chromatography of the extract, the eluent or mobile phase used was n-butanol:acetic acid:water (4:1:5) and using silica gel plate F254 as the stationary phase, the comparison used was routine and obtained an Rf value of 0, 55cm. Test the total flavonoid content of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) by method 2 which is listed in Supplement II of the Indonesian Herbal Pharmacopeia edition I [11]. The maximum wavelength obtained is 411 nm, then the calibration curve is measured with concentrations of 140, 120, 100, 80 and 60 ppm to obtain a linear equation y= 0.0065x – 0.1207 with R = 0.9992 then measure the absorbance of the extract at wavelength of 411 nm, then enter the absorbance value of the extract into a linear equation so that the average total flavonoid content is 1.18%.

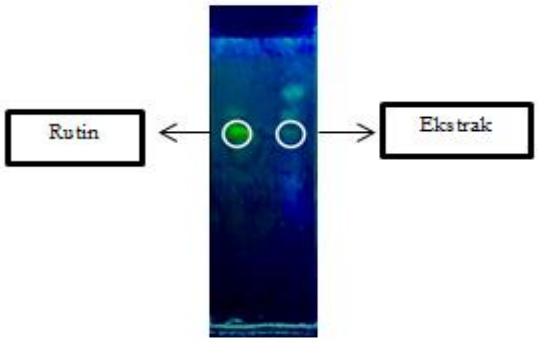

Figure 1. Thin Layer Chromatography of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) viewed under a366 nm UV lamp

Based on the results of the total leukocyte count, the increase in leukocyte cells was found in group 3, namely 12.08 x 103, namely in the treatment of extract administration from day 1-3 induced by vaccine day 4 and continued with extract until day 7. Based on one-way ANOVA statistical test showed that there was a difference significantly from 7 treatment groups (P value <0.05). The results of the calculation of the average total number of leukocytes of male white mice given pegagan embun extract and exposed to H5N1 virus antigen sequentially from group 1 to group 7 were 8.54; 10.06; 12.08; 7.52; 8.26; 5.62; and 6.42 x 103 cells/µL These results were still within the normal range of total leukocyte values in mice, the total number of normal leukocytes in mice ranged from 6x103-15x103 [12]. An increase in the number of leukocytes describes a humoral and cellular response against pathogenic agents or an increase in the body's defense capability [13].

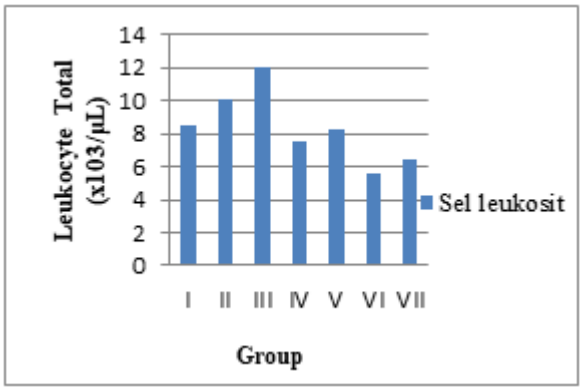

Figure 2. Graph of total leukocyte count of male white mice after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg body weight exposed to H5N1 virus antigen.

The results of the calculation of the percentage of leukocyte cell types in male white mice after the administration of pegagan embun extract, the highest percentage were lymphocytes and the least were eosinophils. As according to the literature, in mice the largest percentage is 70-80% lymphocyte cells and the least is basophils no more than 2% [14]

The percentages of eosinophils sequentially from group I to group VII were 2.20%, 5%, 5.60%, 4.80%, 3%, 2.40% and 5.40%. The results of one-way ANOVA statistical analysis showed a significant difference (p<0.05), eosinophils would increase in number during the response to antigens, parasites and allergies [15]. The percentage of consecutive rod neutrophils was 23.8%, 21.4%, 28.20%, 15.40%, 29.80%, 29.20% and 23.80%, the percentage of consecutive segment neutrophils was 16.8%, 17 .8%, 14%, 28.2%, 14.8%, 23.2% and 14.6%. Based on the results of one-way ANOVA analysis, it was stated that there was a significant difference in stem neutrophil cells (p<0.05) and there was no significant difference in segment neutrophil cells (p>0.05). The normal percentage of neutrophils in mice is 20-30% of the differential leukocyte cell count [16].

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The percentage of lymphocytes sequentially from group I to VII was 39.6%, 37.6%, 36.8%, 40.4%, 39.8%, 34.8%, and 38.4%. After ANOVA analysis was performed. one direction shows p> 0.05 there is no significant difference. In the body, lymphocytes are the second most common type of leukocyte after neutrophils (20-40% of total leukocytes). The number of lymphocytes in children is relatively more than the number of adults, and the number of these lymphocytes will increase when a viral infection occurs [3].

The percentage of monocytes was 17.6%, 18.2%, 15.4%, 11.2%, 13.2, 10.4% and 17.8%, respectively. Based on the one-way ANOVA statistical test there was a significant difference (p <0.05), monocytes are the largest leukocytes and the normal percentage of monocytes in mice ranges from 2-6% of the circulating cell population and their number increases in response to infection [18].

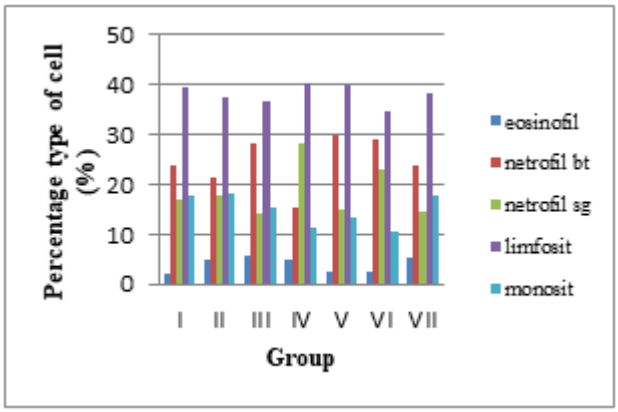

Figure 3. Graph of the percentage of male white mouse cell types after administration of a standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg body weight exposed to H5N1 virus antigen.

IV. CONCLUSIONS

From the results of research that has been carried out on the effect of giving standardized extract of pegagan embun on the total number of leukocytes and the percentage of cell types in male white mice exposed to H5N1 virus antigen, it can be concluded, Giving pegagan embun extract at a dose of 200 mg/kgBW with 7 variations in the timing of antigen administration. can increase the total number of leukocyte cells in male white mice exposed to the H5N1 virus antigen. And can increase the percentage of eosinophil cells, stem neutrophil cells, segment neutrophil cells and monocyte cells. It is suggested that further researchers can use the SARS-CoV-2 virus antigen and determine the active compounds contained in pegagan embun that can increase the activity of NK cells, CD8 cells and leukocyte cells.

ETHICS APPROVAL

Ethics approval was obtained from the ethics committee of the medical faculty of Andalas University. This research has passed the ethical review No: 174/UN.16.2/KEP-FK/2020 letter of ethics.

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