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Original Article

The Activities of Pegagan Embun (*Hydrocotyle sibthorpioides* Lam.) on TNF-α, Macrophages and Leukocytes Male White Mice Exposed by H5N1 Virus Antigens

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ABSTRACT

Introduction: Pegagan embun (*Hydrocotyle sibthorpioides* Lam.) has been known to have immunostimulatory activity, it can increase the activity and capacity of mice macrophage phagocytosis at optimum dose of 200 mg/kgbw. **Aim**: This study aims to determine the activity of the ethanol extract of pegagan embun on TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types. **Methods:** Mice were divided into four groups, one group as control was given 0.5% Na CMC suspension, three groups were given pegagan embun extract at a dose of 10 mg/kgbw, 50 mg/kgbw and 200 mg/kgbw orally for 7 days. On the 8th day, all mice were induced with 0.3 mL H5N1 vaccine subcutaneously, then left for 24 hours. After 24 hours, the mice were sacrificed and then TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types were determined. The research data were analyzed using one-way ANOVA (significance was taken at $p<0.05$) and continued with the Duncan test. **Results:** The results showed that pegagan embun extract significantly reduce TNF-α levels and total macrophages, also significantly increase total leukocytes and percentage of leukocyte types in mice ($p<0.05$). Significant increases and decreases occurred on average at doses of 50 mg/kgbw and 200 mg/kgbw. **Conclusion:** Based on these results, it can be concluded that pegagan embun extract have immunomodulatory activity.

Key words: *Hydrocotyle sibthorpioides* Lam., Immunomodulators, TNF-α, Macrophages, Leukocytes, Leukocytes types.

INTRODUCTION

According to the World Health Organization (WHO), diseases caused by viruses continue to emerge and become a serious problem for world public health. In the last 20 years, there have been 5 zoonotic flu diseases in the world which became a global pandemic and claimed many victims, such as SevereAcute-RespiratorySyndrome (SARS), H5N1 influenza, H1N1 influenza, Middle East Respiratory Syndrome (MERS), and the newest is Coronavirus Disease 2019 (COVID-19) which is still ongoing.[1] All age groups have the potential to be infected with the virus, but there are groups who are more vulnerable, such as the elderly (over 60 years). This is caused by the decline and aging of the function of the immune system which is known as immunosenescence. Immunosenescence and comorbidities in the elderly (hypertension, diabetes, cardiovascular disease, chronic lung disease, and cancer) also significantly increase mortality in this population.[2]

The immune system plays a very important role in protecting the body from viral infections. The immune system is all the mechanisms used by the body to maintain the integrity of the body in order to protect the body from possible dangers posed by microorganisms in the environment. The defense consists of the non-specific (natural/innate) and specific (adaptive/acquired) immune systems. The non-specific immune system is the body's first line of defense against various microorganisms and can respond directly to antigens, while the specific immune system takes time to recognize antigens before it can respond.[3]

Leukopenia, lymphopenia, and proinflammatory cytokines increasement are immunological consequences of viral infection. When infected with a virus, the body responds to tissue damage starting with chemical signals designed to heal the affected tissue. These signals activate leukocyte chemotaxis from the general circulation to damaged tissues. Activated leukocytes will produce cytokines that induce an inflammatory response.[4] Inflammatory cytokines released during the initial response to tissue injury are Tumor Necrosis Factor α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6).[5] The increase in these cytokines results in chemotaxis and accumulation of immune cells (monocytes, macrophages, and T cells) in the lungs, which in turn causes lung damage and respiratory problems. These accumulation of immune cells in the lungs can also cause a cytokine storm. Therefore, one of the therapeutic targets developed for cases of viral infection is immunomodulators.[2]

Pegagan embun (*Hydrocotyle sibthorpioides* Lam.) is known to contain flavonoid compounds which are thought to have immunomodulatory activity. Several flavonoid compounds that have been known to be present in pegagan embun are rutin, quercetin, quercetin 3-(6"-cafeolylgalactoside), genistein, hyperoside, catechin and epicatechin.[6] Pegagan embun has been used traditionally by the community because it has properties such as relieving swelling (anti-swelling), anti-inflammatory, laxative urine, antibiotics, fever reducers, neutralizing toxins (detoxificans), and laxative phlegm (expectorants).[7]

In a study conducted by Afriwardi *et al.* (2021), reported that the extract of pegagan embun has

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anti-inflammatory activity. In this case, the dosage of pegagan embun extract gel is 0.5%; 1%; and 2% topically were able to reduce the volume of exudate in male white mice induced by carrageenan and sesame oil.[8] In a study conducted by Afriwardi *et al.* (2021), it was reported that the ethanolic extract of pegagan embun has an immunostimulant effect that can increase the activity and phagocytic capacity of peritoneal macrophage cells of male white mice with an optimal dose of 200 mg/kgbw.[9]

Based on that explainations, the researchers were interested in conducting research on pegagan embun regarding the activity of the ethanol extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) on TNF-α levels, total macrophages, total leukocytes, and percentage of leukocyte types in male white mice exposed to H5N1 virus antigen. H5N1 virus antigen is used as a substance that will stimulate an immune response. The reason for using the inactivated H5N1 vaccine is because the distribution permit for the SARS-CoV-2 vaccine does not yet exist, so the use of this vaccine has not been allowed by the local health office. In addition, H5N1 influenza has clinical manifestations similar to SARS-CoV-2 infection, one of which is the occurrence of excessive cytokine production.[2,10]

MATERIALS AND METHODS

Tools

The tools used are measuring cylinder glass (Pyrex), erlenmeyer (Pyrex), gavage needle (Terumo), syringe (One Med), rotary evaporator (Buchi R-210 Rotavapor), beaker glass (Pyrex), volume pipette (Pyrex), paper filter (Whatman), dropper, spatula, analytical balance (Ohaus), optical microscope (Motic), UV-Vis spectrophotometer (Genesys 10S UV-Vis), UV lamp (Camag), oven (Memmert), mortar and pestle, glass object (Deckglaser), test tube (Iwaki), TLC plate (Merck), TLC chamber, microtube, centrifuge (Thermo), ELISA reader (BIO-RAD), thermos shaker (Biosan), animal scale, hemacytometer (Neubauer), WBC pipette (Assistant), dark glass bottles, cages for mice, places to eat and drink for mice, vials, scalpels, surgical scissors, gloves (Sensi), masks (Sensi).

Materials

The materials used were pegagan embun (*Hydrocotyle sibthorpioides* Lam.), rutin, aquadest (Andeska Laboratory), 70% ethanol (Andeska Laboratory), sodium carboxy methyl cellulose (Na CMC), physiological NaCl, blue trypan solution, Na-EDTA, Mg powder, HCl P, 2N HCl, FeCl3, Liebermann Burchard reagent (Nitra Kimia), Mayer's reagent (Nitra Kimia), Dragendorff's reagent (Nitra Kimia), AlCl3 P, ethanol P, 1 M sodium acetate, Giemsa dye (Merck), Caprivac AI-K (Caprifarmindo Laboratories), Turk solution (PT. Sagara Husada Mandiri), mouse Tumor Necrosis Factor ELISA kit (BT LAB).

Sample identification

The sample used for this study was pegagan embun (*Hydrocotyle sibthorpioides* Lam.) root, stem, and leaf parts taken from Batu Gadang Village, Lubuk Kilangan District, Padang, West Sumatra, Indonesia. Sample identification was carried out at Andalas University Herbarium (ANDA), Department of Biology, FMIPA, Andalas University, Padang, Indonesia.

Extract preparation

The fresh pegagan embun (*Hydrocotyle sibthorpioides* Lam.) was collected, sorted, washed, chopped, then dried by air-drying in a room not exposed to sunlight.[11] After drying, it was re-sorted, then grinded into powder. The dried pegagan embun powder was weighed 250 g each and put into 4 dark glass bottles and then 2.5 L of 70% ethanol was added. Soaked for the first 6 hours, stirring occasionally, then left for 18 hours. Maserate is separated by filtration. The extraction process was repeated twice with the same type of solvent and the volume of solvent was half the amount of solvent in the first extraction. All filtrate was collected, then evaporated with a rotary evaporator to obtain a thick extract.[12] The thick extract of pegagan embun was weighed according to the planned dose, suspended with 10 mL of Na CMC, then ground until homogeneous. The preparation was given to mice orally by using a gavage needle according to the dose for each mouse.

Extract standardization

Determination of extract quality parameters through standardization which includes standardization of specific parameters and non-specific parameters. The non-specific parameters used in this study were drying shrinkage, total ash content, and acid insoluble ash content. Specific standardization includes organoleptic, phytochemical screening, and determination of crotamogram profile by thin layer chromatography (TLC).

Study design

The animals used were 20 male white mice aged 2-3 months weighing 20-30 g and had never been treated with drugs. Before being used as experimental animals, mice were acclimatized for 7 days to adaptation in new environment, control health, and equate food.[13] These experimental animals were grouped into 4 groups consisting of 5 mice in one group. Group 1 as a control was given 0.5% Na CMC suspension, groups 2, 3, and 4 were given pegagan embun extract at doses of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw orally for 7 days. On the 8th day all mice were induced with 0.3 mL H5N1 vaccine subcutaneously, then left for 24 hours. After 24 hours, the mice were sacrificed and then TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types were determined.

Total leukocytes calculation

Fresh blood was sucked with a leukocyte pipette up to 0.5, then Turk's solution was sucked up to 11, then shaken for 3 minutes. The first and second drops were discarded, then one drop was added to the hemacytometer counting chamber. The liquid is left for 2 minutes for the leukocytes to settle. The number of leukocytes was counted in the four corners of the counting chamber.[3]

$$\text{Total leukocytes} = \text{leukocyte count} \times \frac{20}{0.4}$$

Percentage of leukocyte types calculation

On the 8th day, the tails of mice were cut, dripped with blood on a slide, then flattened with another slide, then dried. After drying, it was dripped with methanol, so that it coats the entire blood smear, left for 5 minutes. After that, stained with Giemsa and left for 20 minutes. Then washed with distilled water, dried and observed under a microscope. Count the number of eosinophils, banded neutrophils, segmented neutrophils, lymphocytes, and monocytes at 100X magnification.[14]

TNF-α levels calculation

Mice were anesthetized with chloroform and blood was taken from the neck circulation by decapitation.[15] Blood was collected into a microtube, left for 30 minutes, then centrifuged at 10,000 rpm for 5 minutes at room temperature. Serum was taken with a micropipette and transferred to a new microtube. Serum was used to measure TNF-α levels using the ELISA method.[16]

Total macrophages calculation

The mice's abdomen was dissected and then 1 mL of physiological NaCl was inserted into the peritoneal cavity of the mice. The mice's abdomen was shaken gently for 2-3 minutes then the peritoneal fluid was pipetted

with a micropipette and put into the tube. 0.5 mL of peritoneal fluid was mixed with 0.5 mL of 0.08% trypan blue solution, then transferred to the hemocytometer counting chamber. Total macrophage cells counting was carried out by counting the number of cells in 80 small squares in the middle.[17] The calculation results were entered into the hemacytometer calculation formula so that total macrophage cells/mm3 was obtained. The calculation formula is as follows:[18]

Total macrophages/mm^3

$$= \frac{1}{CCV} \times \frac{1}{DC} \times \text{macrophages count}$$

Note:

CCV = counting chamber's volume

DC = dilution concentration

Data analysis

The research data were analyzed by one-way ANOVA (significance was taken at $p<0.05$) and continued with Duncan's test using SPSS statistical software version 26.

RESULTS AND DISCUSSION

In this study, samples of pegagan embun were taken from Batu Gadang Village, Lubuk Kilangan District, Padang City, West Sumatra, Indonesia. Before the sample was used, a plant identification test was carried out to avoid errors in selecting the sample. The truth of the identity of the sample is proven by the results of plant identification conducted at the Andalas University Herbarium (ANDA), Laboratory of the Department of Biology, FMIPA, Padang, Indonesia. The results of sample identification showed that the sample used was true pegagan embun (*Hydrocotyle sibthorpioides* Lam.), family *Araliaceae* with identification number 353/K-ID/ANDA/XII/2020.

Extract preparation begins with the collection of 5 kg fresh samples. Furthermore, wet sorting is carried out which aims to separate foreign objects present in the sample and then washed with running water to remove dirt adhering to the samples. After that, the samples were air-dried in a room that was protected from direct sunlight until they became dried simplicia. Then, dry sorting is carried out to separate foreign objects that are still present in the dried simplicia. Dried simplicia was grinded to a powder. The fineness degree of the powder obtained is 8/48 (a bit coarse powder), meaning that 100% of the powder can pass through sieve number 8 and no more than 40% pass through sieve number 48. The size of fineness degree of simplicia will affect the maceration process in terms of extraction obtained perfect and more metabolites are extracted. If the size of the simplicia powder is too large, the contact surface area between the powder and the solvent will be smaller, so that the extraction obtained is less than perfect. Meanwhile, if the size of the simplicia powder is too fine, it will complicate the extraction process because the powder will cover the pores on the filter paper or cotton, causing blockages in the filtering process.[19]

Simplicia powder as much as 1037 g was extracted by maceration method using 70% ethanol as solvent. The selection of maceration as an extraction method aims to avoid damage to chemical compounds that are thermolabile in the pegagan embun. In addition, the maceration method does not require complicated equipment and is easy to work with. The use of 70% ethanol as a filter solution because it has the ability to filter compounds in a wide polarity range from polar to nonpolar compounds, is not toxic compared to other organic solvents, is easier to evaporate than water, is not easily overgrown by microbes, and is relatively inexpensive.[20]

The obtained maserate was filtered with cotton and then evaporated using a rotary evaporator to obtain 170.748 g of thick extracts. The extract yield was calculated by comparing the weight of the thick extract obtained with the weight of the simplicia powder used. The yield of the extract obtained was 16.456%. The higher the yield value, the higher the number of compounds extracted by the solvent.[20]

Determination of extract quality parameters through standardization which includes standardization of specific parameters and non-specific parameters. The non-specific parameters used in this study were drying shrinkage, total ash content, and acid insoluble ash content. Determination of drying shrinkage aims to provide a maximum limit on the amounts of compounds lost in the drying process.[21] The result of determining the drying shrinkage of pegagan embun extract was 4.46%. This shows the amount of water content and compounds lost during the drying process is 4.46%. A good requirement for drying shrinkage is less than 10%, because the drying shrinkage also represents the evaporated water content.[21] Determination of the ash content was carried out to describe the internal and external mineral content from the initial process to the formation of the extract. At this stage the extract is heated until the organic compounds and their derivatives are destroyed and evaporated until only the mineral and inorganic elements remain. The ash content should have a small value because this parameter indicates the presence of heavy metal contamination that is resistant to high temperatures.[21] The result of determining the total ash content of pegagan embun extract was 3.26%. The determination of the acid insoluble ash content was intended to evaluate the extract for contamination of materials containing silica such as soil and sand.[21] The result of the determination of the acid insoluble ash content was 0.2%.

Specific standardization includes organoleptic, phytochemical screening, and determination of crotamogram profile by thin layer chromatography (TLC). Organoleptic observation as a preliminary test, aims to provide an objective and simple initial identification of the extract by utilizing the five senses. The results of organoleptic examination of pegagan embun extract were dark brown in color, thick in consistency, had a characteristic odor, and had a bitter taste.

Phytochemical screening aims to determine the secondary metabolite compounds contained in the extract. The results of phytochemical screening showed that ethanol extract of pegagan embun contained phenolic compounds, flavonoids, saponins, and terpenoids (**Table 1**). These results are in accordance with research conducted by Afriwardi, *et al.* (2021)[8]

Determination of the chromatogram profile by thin layer chromatography (TLC) aims to provide an initial description of the chemical composition based on the chromatogram pattern. Analysis using TLC is the separation of chemical components based on the principle of adsorption and partition determined by the stationary phase (adsorbent) and the mobile phase (eluent). The stationary phase used is silica gel plate GF_{254}. The mobile phase used is butanol: acetic acid: water (4 : 1 : 5). Observations were made under a 366 nm UV lamp after spraying the spot viewer for flavonoids, namely $AlCl_3$.[22] The comparison used is rutin. Rutin with $AlCl_3$ will form a yellow complex

Table 1: Phytochemical screening results.

No.	Secondary metabolite compound	Observation	Result
1	Alkaloids	No white or red precipitate is formed	-
2	Flavanoids	Orange solution	+
3	Phenolic	Dark blue solution	+
4	Saponins	A persistent froth is formed after the addition of HCL	+
5	Terpenoids	A brown ring is formed	+
6	Steroids	No bluish green ring is formed	-

so that at 366 nm UV lamp will see a yellow stain[22] (**Figure 1**). The rutin Rf value obtained was 0.61 and the Rf value of pegagan embun extract was 0.61. It can be concluded that pegagan embun contains rutin compounds.

Mice were grouped into four groups, each of which was given a different treatment, the control group was given 0.5% Na CMC suspension, the test group was given pegagan embun extract at doses of 10, 50, and 200 mg/kgbw. The consideration for choosing the dose was based on previous research on the immunostimulant activity of pegagan embun.[9] Pegagan embun extract has poor solubility in water solvents. Therefore, pegagan embun extract was suspended in 0.5% Na CMC. 0.5% Na CMC is used as a suspending agent because it is inert, non-toxic, non-irritating, and produces a stable solution.[23]

Testing the immunomodulatory activity of pegagan embun extract started by giving the test preparation to each group for 7 consecutive days. On the 8th day, mice were induced with 0.3 mL inactivated H5N1 vaccine subcutaneously and then left for 24 hours. The reason for choosing the H5N1 vaccine as an immune response-inducing agent is because according to research conducted by Nakayama, *et al.* (2012) the H5N1 inactivated vaccine has been shown to stimulate an immune response, one of which is increasing levels of TNF-α.[24] After 24 hours, mice were anesthetized with chloroform and then sacrificed by decapitation and determined the TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types.Then all the research data were tested with one-way ANOVA and continued with Duncan's test.

All doses of pegagan embun extract could significantly increase the total leukocytes of mice ($p<0.05$). In this case, the mice that were given the extract of pegagan embun showed a higher total leukocytes than the control group. The increasement in the total leukocytes is in line with the increasement in the dose. A greater increase in the number of leukocytes was indicated by a dose of 200 mg/kgbw. The average total leukocytes in the control group, the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 4570 cells/mm^3, 7230 cells/mm^3, 8530 cells/mm^3, and 10580 cells/mm^3 (**Figure 2**). These results are in accordance with the research conducted by Afriwardi *et al.* (2021), where the extract of pegagan embun can increase the total leukocytes in mice.[9]

Cells that can be observed in determining the percentage of leukocyte types are eosinophils, banded neutrophils, segmented neutrophils, lymphocytes, and monocytes, while basophils cannot be observed because basophils are alkaline, causing these cells to dissolve when given Giemsa dye. The results of observations of leukocyte types can be seen in **Figure 3**.

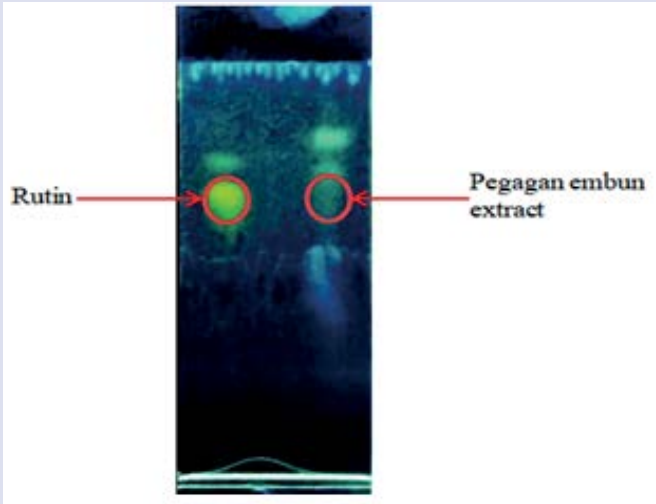

Figure 1: Rutin thin layer chromatogram and pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) after sprayed with $AlCl_3$ under 366 nm UV lamp.

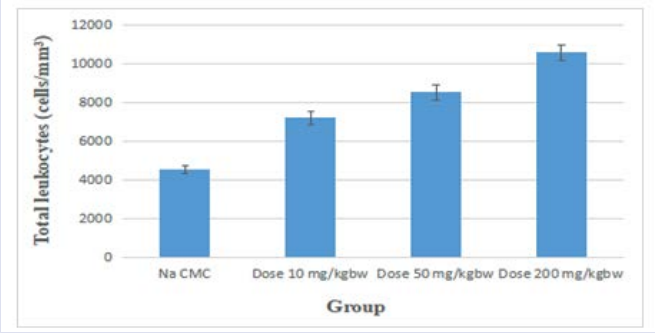

Figure 2: Graph of total leukocytes of male white mice after administration of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) for 7days and then induced by H5N1 virus antigen.

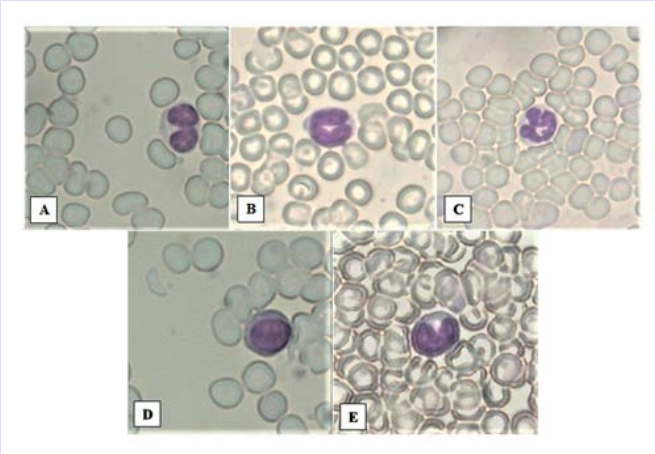

Figure 3: Observation of leukocyte types under a microscope with 100X magnification. **A.** Eosinophil, **B.** Banded neutrophil, **C.** Segmented neutrophil, **D.** Lymphocyte, **E.** Monocyte.

The extract of pegagan embun could significantly increase the percentage of eosinophils in mice ($p<0.05$). In this case, the percentage of eosinophils in mice given the pegagan embun extract was not significantly different, except for the mice given the 200 mg/kgbw were higher than the control group. The average percentage of eosinophils in mice in the control group, in the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 2.8%; 3.2%; 4%; and 5.8% (**Figure 4**). These results are in accordance with the research conducted by Afriwardi *et al.* (2021), where the extract of pegagan embun can increase the percentage of eosinophils in mice.[9]

The extract of pegagan embun could significantly increase the percentage of banded neutrophils of mice ($p<0.05$). In this case, pegagan embun extract significantly increase the percentage of banded neutrophils of mice compared to the control group, except for the mice given the 10 mg/kgbw dose of pegagan embun extract compared to the control group. The average percentage of banded neutrophils in mice in the control group, in the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 4%; 5.4%; 7.6%; and 10% (**Figure 4**). These results are in accordance with the research conducted by Afriwardi *et al.* (2021), where the extract of pegagan embun can increase the percentage of banded neutrophils of mice.[9]

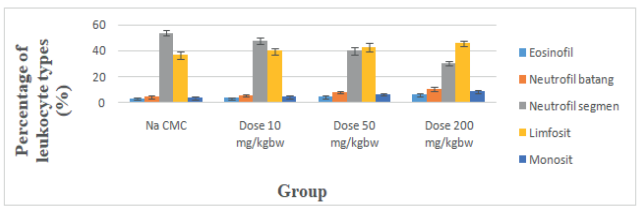

Figure 4: Graph of the percentage of leukocyte types in male white mice after administration of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) for 7 days and then induced by H5N1 virus antigen.

The extract of pegagan embun reduced the percentage of segmented neutrophils in mice significantly ($p<0.05$). In this case, the mice that were given the extract of pegagan embun showed a lower percentage of segmented neutrophils compared to the control group. The percentage of segmented neutrophils decreased with increasing dose. The average percentage of mice segmented neutrophils in the control group, the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 53.6%; 47.6%; 39.8%; and 30.2% (**Figure 4**). The possible cause of the decrease in the percentage of segmented neutrophils is the release of segmented neutrophils from the blood vessels at the time of infection due to chemotaxis. Baratawidjaja & Rengganis (2014) stated, neutrophil cells are only in the circulation for less than 48 hours before migrating and moving very quickly to the area of infection.[25] The decrease in the percentage of segmented neutrophils also occurred in a study conducted by Afriwardi *et al.* (2021).[9]

The pegagan embun extract could significantly increase the percentage of mice lymphocytes ($p<0.05$). In this case, the increase in the percentage of mice lymphocyte significantly occurred in the mice given the pegagan embun extract at a dose of 50 mg/kgbw compared to the control group, although the increase was not significantly different compared to the doses of 10 mg/kgbw and 200 mg/kgbw. The average percentage of mice lymphocyte in the control group, the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 36.6%; 39.6%; 42.6%; and 45.8% (**Figure 4**). These results are in accordance with the research conducted by Afriwardi *et al.* (2021), where the extract of pegagan embun can increase the lymphocyte percentage of mice.[9]

The pegagan embun extract can significantly increase the percentage of mice monocytes ($p<0.05$). In this case, pegagan embun extract could significantly increase the percentage of monocytes in mice compared to the control group, except for mice that were given a dose of 10 mg/kgbw of pegagan embun extract, which was not significantly different from the control group. The average percentage of mice monocytes in the control group, the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 3.0%; 4.2%; 6.0%; and 8.2% (**Figure 4**). These results are in accordance with the research conducted by Afriwardi *et al.* (2021), where the extract of pegagan embun can increase the percentage of mice monocytes.[9]

The extract of pegagan embun could significantly reduce the total macrophages in mice ($p<0.05$). In this case, the mice that were given the pegagan embun extract at doses of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw showed lower total macrophages than the control group. The decrease in total macrophages in line with the increasing dose. The lower total macrophages was indicated by a larger dose of 200 mg/kgbw. The average total macrophages in mice in the control group, in the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, was 6690 cells/mm^3, 3500 cells/mm^3, 2710 cells/mm^3, and 2000 cells/mm^3 (**Figure 5**)

The extract of pegagan embun could significantly reduce the TNF-α levels in mice ($p<0.05$). In this case, the mice given the extract of pegagan embun showed lower TNF-α levels than the control group. Lower TNF-α levels were indicated by a larger dose of 200 mg/kgbw. There was no difference in TNF-α levels between mice that were given a dose of 10 mg/kgbw and a dose of 50 mg/kgbw. The average TNF-α levels in mice in the control group, in the extract group at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw, respectively, were 480.2 ng/L; 246.1 ng/L; 231.6 ng/L; and 221.7 ng/L (**Figure 6**).

In a previous study it was reported that the extract of pegagan embun showed an anti-inflammatory effect. In this case, the administration of the gel preparation of pegagan embun extract at a dose of 0.5%; 1%; and 2% topically were able to reduce the volume of exudate in male white mice induced by carrageenan and sesame oil.[8] Virus antigens or inflammation-inducing substances will activate macrophage cells. Active macrophage cells will produce various cytokines, one of which is TNF-α to support its function in phagocytosis and as an antigen presenting cell (APC). TNF-α will increase the permeability of blood vessels, causing adhesion molecules, neutrophils, macrophages, and plasma proteins to move towards the site of infection to fight the incoming antigen.[25] The accumulation of phagocytic cells and plasma proteins will cause edema. In this research, pegagan embun extract was proven to reduce TNF-α levels of male white mice induced with H5N1 virus antigen. In another study, the extract of pegagan embun showed immunostimulant activity that could increase the activity and phagocytic capacity of peritoneal macrophage cells of male white mice with an optimal dose of 200 mg/kgbw.[9] In addition to increasing vascular permeability, low levels of TNF-α can also increase the activity and phagocytic capacity of macrophage cells. TNF-α in low levels has beneficial effects, but in very large levels it can aggravate

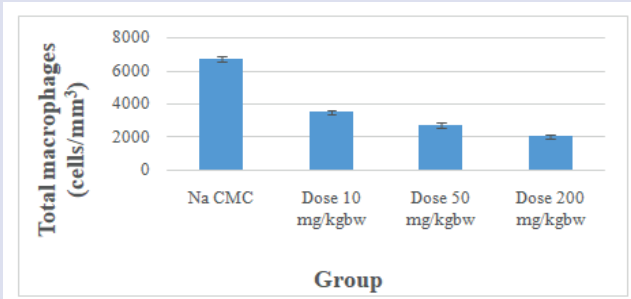

Figure 5: Graph of total macrophages of male white mice after administration of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) for 7 days and then induced by H5N1 virus antigen.

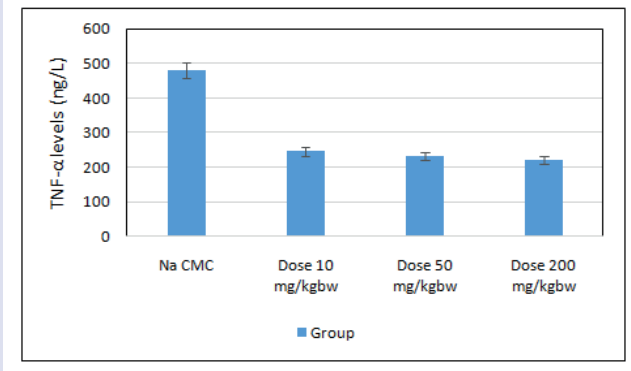

Figure 6: Graph of TNF-α levels in male white mice after administration of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) for 7 days and then induced by H5N1 virus antigen.

inflammatory conditions and cause tissue damage.[26] Based on these results, it can be concluded that the extract of pegagan embun has an immunomodulatory effect.

The increase in the total leukocytes and percentage of leukocyte types also the decrease in the total macrophages and TNF-α levels depended on the dose of the pegagan embun extract given. If the dose is increased, the number of secondary metabolites that act as immunomodulators will increase so that the effect will be better. The increase in the total leukocytes and percentage of leukocyte types was thought to be due to the extract of pegagan embun containing flavonoid compounds. The mechanism by which flavonoids can increase the total leukocytes and percentage of leukocyte types is not yet known because there are no related studies that explain the possible mechanism. However, in the research that has been done, some plants containing flavonoid compounds have been shown to increase the total leukocytes and percentage of leukocyte types in mice, such as research conducted by Afriwardi *et al.* (2021), extracts of pegagan embun at doses of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw can increase the total leukocytes and percentage of leukocyte types in mice induced by *Staphylococcus aereus*.[9] In a study conducted by Aldi *et al.* (2016), basil leaf extract doses of 10 mg/kgbw, 50 mg/kgbw, and 100 mg/kgbw can increase the total leukocytes and percentage of leukocyte types in mice.[3]

The decrease in the total macrophages and TNF-α levels was thought to be due to the extract of pegagan embun containing flavonoid compounds. The flavonoids known to be contained in the pegagan embun extract are genistein, quercetin, rutin, hyperoside, quercetin 3-(6"-cafeolilgalactoside), catechins, and epicatechin.[6] In a study conducted by Comalada *et al.* (2006), quercetin and genistein can inhibit the proliferation of macrophage cells.[27] If macrophage cell proliferation is inhibited, the total macrophage cells in the tissue will be less. Quercetin and genistein can also inhibit TNF-α secretion more effectively (>60% inhibition).[27] Quercetin can also reduce phosphorylation of IκB-α. TNF-α is regulated by the transcription factor NFκB. Under quiescent conditions, NFκB is sequestered in the cytosol bound to the inhibitory protein IκB-α. Cell exposure to LPS triggers a phosphorylation cascade that ultimately leads to phosphorylation and degradation of IκB-α. After IκB-α is released from the complex, NFκB translocates into the nucleus where it binds to specific DNA motifs in the promoter region, leading to an increase in TNF-α transcription.[27] When phosphorylation of IκB-α is reduced, TNF-α levels can be reduced. In addition, quercetin can also induce the secretion of IL-10 which is an anti-inflammatory cytokine.[27] If IL-10 levels increase, inflammatory cells can be reduced. According to Santangelo *et al.* (in Serafini *et al.*, 2010), quercetin and catechins combine their inhibitory action on TNF-α and IL-1β to increase the release of the anti-inflammatory cytokine IL-10.[28] In the study conducted by Nam et al. (2017) it is known that the main flavonoid contained in tartary buckwheat sprout extract (TBS) is rutin in large amounts. TBS extract showed higher inhibitory activity as assessed by the production of proinflammatory mediators such as nitric oxide and cytokines including TNF-α, IL-6, and IL-12 in LPS-stimulated RAW 264.7 macrophages. In addition, TBS extract suppressed NFκB activation by preventing IκB-α degradation and MAPK phosphorylation in LPS-stimulated RAW 264.7 macrophages. In addition, TBS extract markedly reduced LPS-induced cytokine production in peritoneal macrophages.[29]

Beside containing flavonoids, pegagan embun also known to contain terpenoid asiaticoside and madecoside compounds.[6] These compounds are the main ingredients in the pegagan (*Centella asiatica* L.). Based on research conducted by Punturee *et al.* (2005), the water extract of pegagan (*Centella asiatica* L.) showed immunostimulant activity, in this case the water extract of pegagan (*Centella asiatica* L.) could increase the levels of IL-2 and TNF-α in peripheral blood mononuclear cells (PBMC) in humans in vitro. In contrast to the water extract of the pegagan (*Centella asiatica* L.), the ethanolic extract of pegagan (*Centella asiatica* L.) actually showed immunosuppressant activity, in this case the water extract of pegagan (*Centella asiatica* L.) could decrease the levels of IL-2 and TNF-α on human Peripheral blood mononuclear cells (PBMC) *in vitro*. However, the mechanism of the extract of pegagan (*Centella asiatica* L.) has unknown immunomodulatory activity.[30] There are still few scientific publications regarding the immunomodulatory activity of the pegagan (*Centella asiatica* L.) and the immunomodulatory activity of the isolation of these asiaticoside and madecoside compounds. Therefore, it is recommended for further researchers to examine what class of chemical compounds in the pegagan embun (*Hydrocotyle sibthorpioides* Lam.) has the most optimal immunomodulatory activity, both flavonoids, terpenoids, and other chemical compounds.

CONCLUSION

Based on the results of research that has been conducted regarding the activity of pegagan embun extract on TNF-α levels, total macrophages, total leukocytes, and percentage of leukocyte types, it can be concluded that Ethanol extract of pegagan embun could reduce TNF-α levels and total macrophages of male white mice exposed to H5N1 virus antigen. The ethanol extract of pegagan embun could also increase the total leukocytes and the percentage of eosinophils, banded neutrophils, lymphocytes, and monocytesin male white mice exposed to the H5N1 virus antigen.

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CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

SUMMARY

Pegagan embun (*Hydrocotyle sibthorpioides* Lam.) has been known to have immunostimulatory activity, it can increase the activity and capacity of mice macrophage phagocytosis at optimum dose of 200 mg/kgbw. This study aims to determine the activity of the ethanol extract of pegagan embun on TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types. Mice were divided into 4 groups, one group as control was given 0.5% Na CMC suspension, three groups were given pegagan embun extract at a dose of 10 mg/kgbw, 50 mg/kgbw, and 200 mg/kgbw orally for 7 days. On the 8th day, all mice were induced with 0.3 mL H5N1 vaccine subcutaneously, then left for 24 hours. After 24 hours, the mice were sacrificed and then TNF-α levels, total macrophages, total leukocytes, and percentage of leukocytes types were determined. The research data were analyzed using one-way ANOVA (significance was taken at $p<0.05$) and continued with the Duncan test. The results showed that pegagan embun extract significantly reduce TNF-α levels and total macrophages, also significantly increase total leukocytes and percentage of leukocyte types in mice ($p<0.05$). Significant increases and decreases occurred on average at doses of 50 mg/kgbw and 200 mg/kgbw. Based on these results, it can be concluded that pegagan embun extract have immunomodulatory activity.

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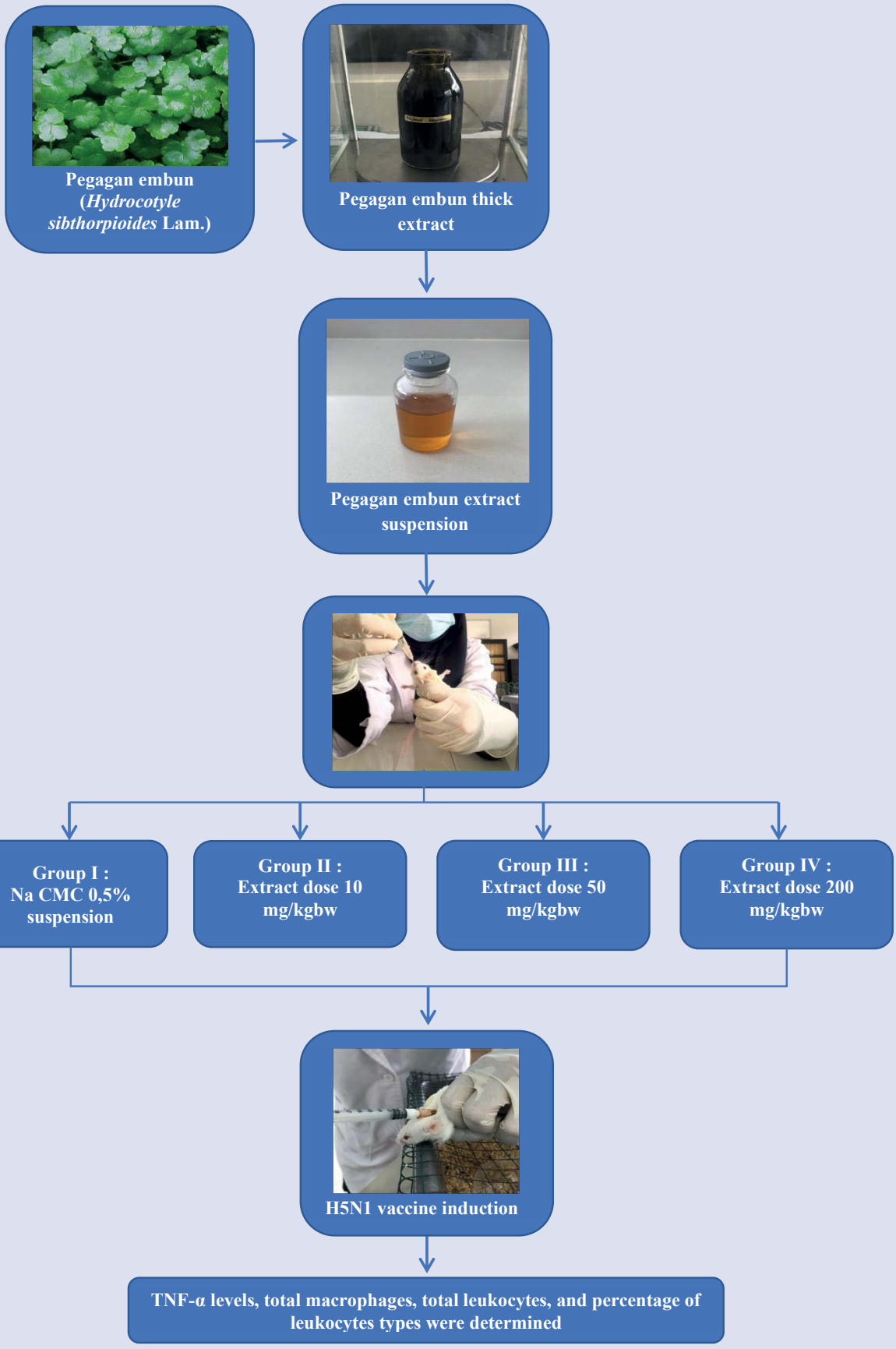
GRAPHICAL ABSTRACT



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