

# Effect of Standardised Extract Pegagan Embun (Hydrocotyle sibthorpioides Lam). toward Natural Killer Cell and CD8 Cell Activities on While Male Exposed to H5N1 Virus Antigen

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## Effect of Standardised Extract Pegagan Embun (*Hydrocotyle sibthorpioides* Lam.) toward Natural Killer Cell and CD8 Cell Activities on White Male Mice Exposed to H5N1 Virus Antigen

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### Abstract

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**AIM:** Community in Indonesia used pegagan embun (*Hydrocotyle sibthorpioides* Lam.) to increase endurance. Based on that, the study aimed to determine the effects of pegagan embun extract on natural killer (NK) cells and CD8 cells activities in white male mice exposed to the H5N1 antigen.

**MATERIALS AND METHODS:** As many as thirty-five experimental white male mice were used, divided into seven groups, by varying the time of administration of the H5N1 antigen. Groups I and II, were given the test extract for 7 days and induced H5N1 antigen on days 1 and 7, then evaluated on day 8. Group III was given a test extract for 3 days, then induced antigen on the 4<sup>th</sup> day and continued with giving the test extract until day 7<sup>th</sup>. In Groups V and VII, only H5N1 antigen induction on day 1 and evaluated on day 8. Groups IV and VI were given the test extract for 7 and 4 days. The observations examine the activities of NK cells and CD8 cells. The data were analyzed with a one-way analysis of variation method with  $p = 0.05$ , then analyzed with Duncan Multiple Range Test.

**RESULTS AND DISCUSSION:** After calculating, the NK cell activity for groups 1 to 7, respectively: 2.12; 2.03; 2.07; 1.87; 1.98; 1.91, and 1.95 ng/mL while for CD8 cell activity 22.23; 24.61; 23.69; 21.10; 19.20; 19.87, and 18.13 ng/mL. The results showed that giving pegagan embun extract to white male mice exposed to the H5N1 antigen increase the activities of NK cells and CD8 cells.

**CONCLUSION:** It concluded that administration of standardized extract of pegagan embun (*H. sibthorpioides* Lam.) at a dose of 200 mg/kg BW increased NK cells activities and CD8 cells of white male mice exposed to H5N1 virus antigen.

### Introduction

The immune-mediated disease is an enormous problem in developing countries such as Indonesia. Viruses, bacteria, fungi, protozoa, and parasites are some of the most common pathogenic microorganisms found in the environment, and they are the principal causes of illness in people [1].

The immune system is a defense system in the human body separated into the nonspecific immune system and specific immune system [2]. The nonspecific immune system is the immune system acquired from birth, while the specific immune system is the acquired immune system [3]. The nonspecific immune system fights disease in the same way against all types of disease. It works quickly if pathogenic microorganisms enter the body. In contrast, the specific immune system works precisely because the response to each microbe is different and must recognize the type of microbe to be treated. Therefore, this immune system works for a long time to cause a response [2].

In the body, nonspecific and specific immune systems work together to eliminate the infection. The immune response consists of various cells and soluble molecules secreted by these cells. The primary cells such as lymphocytes (B cells, T cells, and natural killer [NK] cells), phagocytes (neutrophils, eosinophils, monocytes, and macrophages), accessory cells (basophils, mast cells, and platelets) involved in immune reactions on the infection [4]. NK cells are part of lymphocyte cells where NK cells can kill target cells directly without prior sensitization and without depending on major histocompatibility complex (MHC). Apart from being independent of MHC, these cells also do not interact with target cells via the T cell receptor (TCR).

NK cells have an essential role in the natural defense against the growth of cancer cells and various infectious diseases, especially viral infections. 95% NK cells functioned as killer cells. The virus-infected target cells and other target cells coated with immunoglobulin G (IgG). IgG-coated virus-infected target cells and other target cells (IgG). As a result, NK cells are cytotoxic

cup (Pyrex), measuring flask (Pyrex), volume pipette (Pyrex), digital scale (SF-400), Silica gel 60 F254 (Merck), desiccator, dark bottle, TLC vessel, animal cage, oven (Memmert), centrifuge (Gemmy PLC-03), hemocytometer (Neubauer), spectrophotometer (BIO-RAD), slides (Slides), microplate, Elisa reader (BIO-RAD), incubator (Biosan).

### Materials

The materials used pegagan embun, aquadest (Andeska), Na Charboxy Methyl Cellulosa, 70% ethanol (Andeska), 80% ethanol, ethyl alcohol P (Merck), Chloroform P (Merck), Methyl alcohol P (Merck), glacial acetic acid P (Merck), routine, silica gel 60 F254 (Sentana), KI (Merck), Feri Clorida (Merck), Hidragiry Clorida (Merck), Alumunium Clorida (Merck), Mouse NK cell kit and CD8 cells (Bioassay Technology Laboratory), H5N1 Vaccine no. batch VF09B30 (Caprivac® AI-K).

### Collecting and identification of pegagan embun

Pegagan embun sample collected in Batu Gadang Village, Lub Kilangan District, Padang City, West Sumatra. The ANDA Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA) Universitas Andalas (UNAND) Padang, West Sumatra, Indonesia was identified this pegagan embun.

### Extracting pegagan embun

A total of 1 kg of pegagan embun was dried and finely ground before being macerated in a 70% ethanol solvent. Fill the macerator with one part dry Simplicia powder and ten parts solvent. Soak for the 6 h, stirring occasionally, and then strain after 18 h. Repeat the filtering process twice more, using the same type and amount of material each time. Collect all of the macerates and evaporate using a rotary evaporator until thick extract obtained.

### Extract standardisation

Pegagan embun extracts were characterized in both nonspecific and particular ways. Drying shrinkage, total ash content, and acid insoluble ash content were used for nonspecific characterization, whereas an organoleptic test, identity parameter, chemical content test, thin-layer chromatography, and total flavonoid content determination were used for specific characterization.

### Dosage and induction

Dosage for induction used one variant optimal dose of pegagan embun extract as 200 mg/kg BW [21]. The induction used the H5N1 Cavrivac® AI-K vaccine with a dose of 50 µL administered intramuscularly.

### Treatment of experimental animals

This study used healthy white male mice (*Mus musculus* BALB/c strain) with a bodyweight of 22-28 g. Totalling thirty-five white male mice divided randomly into each group consisting of five mice. Table 1 shows the treatment of this study.

Table 1: The group's treatment

Group	Extract induced (day)	Vaccinated (day)	Evaluation (day)
I	1 <sup>st</sup> -7 <sup>th</sup>	7 <sup>th</sup>	8 <sup>th</sup>
II	1 <sup>st</sup> -7 <sup>th</sup>	1 <sup>st</sup>	8 <sup>th</sup>
III	1 <sup>st</sup> -3 <sup>rd</sup>	4 <sup>th</sup>	8 <sup>th</sup>
	4 <sup>th</sup> -7 <sup>th</sup>	-	-
IV	2-7 (Na CMC)	1 <sup>st</sup>	8 <sup>th</sup>
V	1 <sup>st</sup> -7 <sup>th</sup>	-	8 <sup>th</sup>
VI	2 <sup>nd</sup> -4 <sup>th</sup>	1 <sup>st</sup>	8 <sup>th</sup>
VII	1 <sup>st</sup> -4 <sup>th</sup>	-	5 <sup>th</sup>

The treatment is given once a day in the afternoon. Before being treated, the experimental animals were acclimatised for 7 days. The treatment process lasted for 7 days, and an evaluation was eight mornings [22]. The experimental animals were provided feed and water, then their body weight and motoric activities were monitored every day. The experimental animals were those whose body weight did not surpass 10% of the average experimental animal's body weight.

### Blood serum collecting for NK cells and CD8 cells

Blood drawn utilizing the guillotine (neck artery). Then the blood was collected and centrifuged for 30 min at 3000 rpm. Then the serum was used to test the levels of NK cells and CD8 cells using the ELISA method. The protein markers from NK cells and CD8 can be detected in plasma cells using an ELISA kit [23].

### Data analysis

The data were analyzed statistically with the One-way analysis of variation (ANOVA) method. Then, the result was analyzed with Duncan's Multiple Range Test with IBM SPSS type 24.

### Ethics approval

The ethics approval on animal testing was obtained from the Ethics Committee of the Faculty of Medicine, Universitas Andalas. This research passed the ethical review with letter of ethics contract number: 174/UN.16.2/KEP-FK/2020.

### Results

The chromatography of pegagan embun extract (*H. sibthorpioides* Lam.) showed in Figure 1:

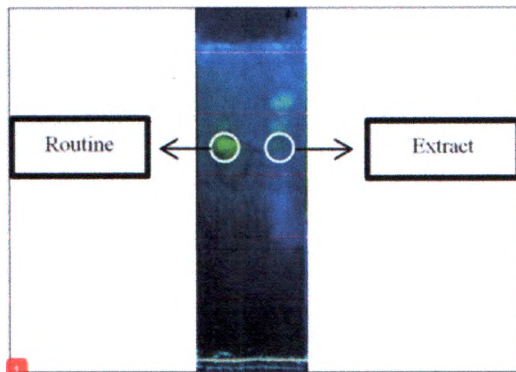


Figure 1: Thin layer chromatography of pegagan embun extract (*Hydrocotyle sibthorpioides* Lam.) viewed under a 366 nm UV lamp

The concentrations of NK cells and CD8 cells showed in (Figures 2 and 3).

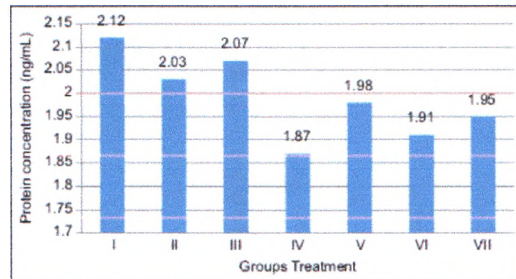


Figure 2: Graphic of NK cell protein concentration of white male mice after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

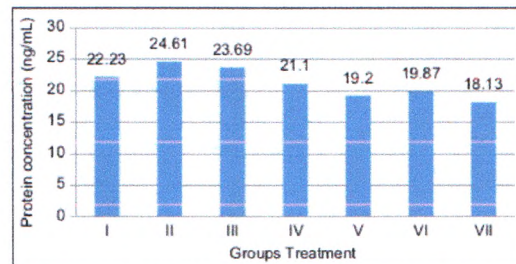


Figure 3: Graphic of CD8 cell protein concentration of white male mice after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

The ANOVA test and DMRT on NK cells conducted, and the result showed in Tables 2 and 3:

Table 2: The result of ANOVA Test of NK Cells activities after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

	Sum of square	Df	Mean Squares	F Ratio	Sig.
Between Group	0.22	6	0.03	2.72	0.033
Within Group	0.39	28	0.01		
Total	0.61	34			

Table 3: The result of DMRT of NK Cells activities after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

Groups	n	Subset for alpha = 0.05		
		I	II	III
4	5	1.87		
6	5	1.91	1.91	
7	5	1.95	1.95	
5	5	1.98	1.98	1.98
2	5	2.03	2.03	2.03
3	5	2.07	2.07	2.07
1	5			2.12
Sig.		0.07	0.07	0.09

The ANOVA test and DMRT on NK Cells conducted, and the result showed in Tables 4 and 5:

Table 4: The result of ANOVA Test of CD8 activities after administration of standardized extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

	Sum of square	Df	Mean squares	F Ratio	Sig.
Between group	170.39	6	28.39	18.35	0.000
Within group	43.31	28	1.54		
Total	213.70	34			

Table 5: The result of DMRT of CD8 Cells activities after administration of standardised extract of pegagan embun (*Hydrocotyle sibthorpioides* Lam.) 200 mg/kg BW exposed to H5N1 virus antigen

Groups	n	Subset for alpha=0.05					
		I	II	III	IV	V	VI
7	5	18.13					
5	5	19.2	19.2				
6	5	19.86	19.86	19.86			
4	5			21.09	21.09		
1	5			22.23	22.23	22.23	
3	5			23.69	23.69	23.69	23.69
2	5					24.61	24.61
Sig.		0.18	0.40	0.13	0.16	0.07	0.25

## Discussion

The extract chromatographed a very thin layer using n-butanol: acetic acid: water (4:1:5) as the eluent and silica gel plate F254 as the stationary phase, using rutin as the comparison and an Rf value of 0.55cm. Pegagan embun (*H. sibthorpioides* Lam.) total flavonoid content by method two, as published in Supplement II of the Indonesian Herbal Pharmacopeia version I (Ministry of Health, 2011) 411 nm the maximum wavelength obtained. The calibration curve 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 the absorbance of the extract measured at 411 nm, and the absorbance value of the extract entered into a linear equation to yield 1.18% average total flavonoid content.

Fresh pegagan embun collected, cleaned of contaminants, washed with running water, and air-dried to become dry *Simplicia* before being used to make pegagan embun extract. *Simplicia* dried, then mashed in a blender to get powdered *Simplicia*. A 70% ethanol solvent was used to macerate the samples [24]. As much 170.74 grams of extract recovered from 1037.05

grams of *Sibthorpioides* powder, with an extract yield of 16.46%. The drying shrinkage of the extract was 3.95% after standardisation of pegagan embun extracts. This conclusion was consistent with the Indonesian Herbal Pharmacopeia, which states that the drying shrinkage of pegagan embun extract is less than 10%. The total ash content of pegagan embun extract (*H. sibthorpioides* Lam.) was determined to be 2.55%. It met the requirements of the Indonesian Herbal Pharmacopeia, which stated that the total ash content of pegagan embun extract should not exceed 16.6%. According to the Indonesian Herbal Pharmacopeia, the acid insoluble ash content produced is 0.07%. The extract of pegagan embun contains no more than 2.3% insoluble ash.

The organoleptic study designed to provide a basic introduction to the pegagan embun extract, which has a dark brown color and a bitter taste. The phytochemical test revealed that the pegagan embun extract contained flavonoids, phenolics, terpenoids, and saponins. The serum sample was used to examine NK cells and CD8 cells, obtained from centrifugation of mice blood taken from the neck of mice and then the concentration determined by the ELISA method. NK cells and CD8 cells function to kill cells that are infected and unwanted by the body [25]. The highest concentration of NK cells was found in Group 1, which was 2.12 ng/ $\mu$ L, and the lowest concentration in Group 4 in the treatment group induced by vaccine without extract, which was 1.87 ng/ $\mu$ L. After the one-way ANOVA statistical, there was a significant difference with a  $p < 0.05$ . It explained that the data from each treatment group indicated an increase in the concentration of NK cells compared to the group of mice induced by the vaccine. NK cells play an essential role in nonspecific immunity against intracellular pathogens that can recognize and kill abnormal cells and destroy cells that contain viruses or neoplasm cells. NK cells are activated by interferons which are generally produced and released by virus-infected cells. Interferons affect accelerating maturation and cytolytic effects of NK cells. It increased virus resistance in uninfected cells [21], while NK cells are a subset of lymphocytes with either a CD 16 or CD 56 surface (a receptor for FC). The surface characteristics of CD 16 and 56 have been used to ensure that these cells are NK cells that can differentiate between T cells and B cells. It also does not interact with the TCR [26].

NK cells components of innate immunity act as killers (cytotoxicity) by secreting lysosomes containing perforins and granzymes and producing cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-5, IL-13 [27]. NK cells also function as co-stimulatory that can stimulate macrophages, T cells, and B cells, thus bridging the interaction between innate immunity and adaptive immunity [28]. The concentrations of NK cells and CD8 cells showed in (Figures 2 and 3).

The graphic showed the highest CD8 cell protein weight in Group II, 24.61 ng/mL. The group that

received the extract treatment for 3 days then induced on the 4 days and continued with the extract until day eighth compared to Group 7 that could treat only induced by the vaccine on the 1<sup>st</sup> day and evaluated on day 4<sup>th</sup> which was 18.13 ng/mL, and this proved that there was an increase in the concentration of CD8 cells in the group that was given the extract and induced with the H5N1 vaccine compared to the group that only induced without given extract.

ANOVA result showed a significant difference ( $p < 0.05$ ). CD8 cells express the CD8 co-receptor and destroy infected cells between dependent MHC-I specific antigens. CD8 cells can kill cells directly and through the induction of apoptosis [29]. CD8 cells contain abundant azurophilic granules capable of destroying various tumor cells, infected cells, and abnormal cells without prior sensitization [30]. The innate and adaptive immune systems carry out an effective viral response from the host through the production of various proinflammatory cytokines, activation of T cells, CD 4, and CD8 cells. T cells are essential for controlling viral replication, limiting virus spread, and clearing infected cells. However, tissue caused by viruses makes excessive production of proinflammatory cytokines, recruitment of proinflammatory macrophages and granulocytes. It is called a Cytokine Storm, leading to more severe tissue damage [31].

## Conclusion

This study showed administration of standardized extract of pegagan embun (*H. sibthorpioides* Lam.) at a dose of 200 mg/kg increases NK cells activities and CD8 cells of white male white mice exposed to H5N1 virus antigen. The body's ability to kill viruses that enter has been enhanced due to the increase activity of NK cells and CD8 cells. Following investigations should undertake acute and subacute toxicity tests so that this standardized extract may be examined clinically.

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