Immunostimulatory Activities of Pegagan Embun (Hydrocotyle sibthorpioides Lam.) in White Male Mice

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Immunostimulatory Activities of Pegagan Embun (*Hydrocotyle sibthorpioides* Lam.) in White Male Mice

Afriwardi[1,*], Yufri Aldi[2], Dwisari Dillasamola[2], Yolanda Alfiyyah Larakhansa[2], Elsa Badriyya[2]

ABSTRACT

Introduction: Pegagan embun (*Hydrocotyle sibthorpioides* L.) has many pharmacological activies, such as improving the immune system. **Aim:** This research aims to study the immunomodulatory effect of *pegagan embun* herbs ethanol extract (*Hydrocotyle sibthorpioides* L.) determined by phagocytic activity and capacity of macrophages, total and percentage of leukocytes. **Methods:** 25 male white mice were divided into 5 equal groups. Negative control group were given Na CMC 0.5%, the extract treated group were given *pegagan embun* ethanol extract at dose of 10, 50, 200 mg/kgbw, and the positive control group was given Stimuno 50 mg/kgbw orally for 7 days. On the 8th day, the total and percentage of leukocytes were counted through blood sample taken intravenously. The mice were then induced with *Staphylococcus aureus* suspension. After one hour, the peritoneal fluids was taken to determine the macrophages activity and capacity. The macrophages phagocytic activity and capacity, total and percentage of leukocytes were analyzed by One-Way Anova followed by Duncan Multiple Range Test ($p<0.05$). **Results:** The results show significant increase of concentration ($p<0.05$) towards macrophages phagocytic activity and capacity, and total leukocytes count. Percentage of leukocytes show that lymphocytes increase significantly ($p<0.05$), meanwhile neutrophils segments decrease significantly ($p<0.05$). **Conclusion:** It can be concluded that ethanol extract of *pegagan embun* herb at doses of 10, 50, 200 mg/kgbw shows immunostimulatory activity.

Key words: *Hydrocotyle sibthorpioides* Lam., *Staphylococcus aureus*, Phagocytosis, Macrophage, Leukocytes.

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INTRODUCTION

The immune system protects human from foreign substances and pathogens such as viruses, bacteria, parasites, and fungi. There are two types of immune response such as specific and non-specific immune response. Non-specific immune response works rapidly to protect human from microorganisms while specific immune response shows specific response towards specific microbes.[1,2]

Non-specific immune response protect the body by phagocyting antigen such as bacteria without regarding its difference from other foreign substances. The most important phagocytic cells in this process is the macrophages. Macrophages come from adult monocytes in the tissues. The two main functions of marophages are destroying antigens and presenting them to lymphocytes T as macrophages acts as Antigen Presenting Cells (APC).[3]

An immunostimulant can increase body defense mechanism. Generally, an immunostimulant is defined as a compound that can increase body defense mechanism specifcally and non-specifically through cellular or humoral response.[3] Certain plants contains compound that show immunostimulatory activity. One of those plants is known as *pegagan embun* (*Hydrocotyle sibthorpioides* Lam.) herbs that is widely used in Chinese traditional medicines to treat immune and liver related disease.[4] *Pegagan embun* has several medicinal properties such as anti-swelling, anti-inflamatory, diuretics, antibiotics, antipiretics, detoxificans, and expectorants. Farong Yu *et al.* reported that *Hydrocotyle sibthorpioides* extract shows excellent anti-tumour activities and helps repairing mice's immunologic functions.[6]

Based on the above explainations, a research to determine the immunomodulatory activity of *Hydrocotyle sibthorpioides* extract was conducted. The determined parameters were macrophages phagocytic activity and capacity, total leukocytes count, and percentages of leukocytes in white male mice induced by *Stapylococcus aureus*.

MATERIALS AND METHODS

Time and place

This study was carried out for 4 months at Research Laboratorium and Immunology and Serology Laboratrium of Faculty of Pharmacy of Andalas University.

Apparatus

The apparatus used in this study were measuring cylinder (Pyrex), erlenmeyer (Pyrex), gavage needle (Terumo), rotary evaporator (Ika), filter paper (Whatman), analitical balance (Ohaus), microscope (Olympus), TLC plate (Merck), centrifuge, WBC pipette (Assistant), mice cage, and surgical scissors.

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Materials

The materials used in this study were *pegagan embun (Hydrocotyle sibthorpioides Lam.)*, stimuno (Dexa Medica, Batch No. : SLBZ4289), quercetin (Sigma-Aldrich, CAS Number: 117-39-5), aquadest (Andeska Laboratory), carbomethylcellulose (Na CMC), Giemsa stain 0,5% (Merck), ethanol 70% (Andeska Laboratory), *Staphylococcus aureus* (Microbiology Laboratory of Faculty of Pharmacy of Andalas University), nutrient agar (Merck), nutrient brooth (Merck), physiologic NaCl (Andeska Laboratory), Turk solution (Sagara Husada Mandiri), and white male mice (Wistar).

Extract preparation

1 kg of dried *pegagan embun* were finely grinded (Mesh 20) and macerated with ethanol 70%. An amount of fine dried powder and solvent were added into a mecerator with a ratio of 1:10. The mixture was strirred occasionally for 6 hours, left for 18 hours, and then filtered. The above processes were repetead two times with the same ratio of dried powder and solvent. The macerates were collected and concentrated by rotary evaporator until a crude extract was obtained.[7] The extract was tested before use. The tested parameters were organoleptic, phytochemical screening, and TLC profile.

Animal model

The animal used in this research was 25 naive white male mice, 2-3 months old that weigh 20-30 g. The test animal were grouped into 5 groups consisted of 5 animals each. The negative control group was given Na CMC 5% suspension, group 2,3, and 4 were given *pegagan embun's* extracts with doses of 10, 50, and 200 mg/kgbw while positive control group was given stimuno with doses of 50 mg/kgbw. The extract was given orally for 7 days.

Bacteria culture

Staphylococcus aureus R. (SA) was cultured into slanted nutrient agar (NA) and then innoculated into a new NA using the innoculating needle. Then the bacteria was incubated for 24 hours at 37° C. *Staphylococcus aureus* R. was then moved into the nutrient broth (NB) and incubated at 37° C for 24 hours. It was then centrifuged at 2500 rpm for 25 minutes until a pellet was formed and suspended with physiological NaCl.[8]

Total leucocyte count

Fresh blood was taken using WBC pipette until the number 0.5 and turk solution was taken until the number 11 then shook for 3 minutes. The first 2 drops of the solution in the pipette were removed. Then, 1 drop of the solution was dropped on the hemocytometer. Leave for 2 minutes for the leukocytes to be sedimented. The white blood cells were counted at the 4 corner of the counting chamber.[9]

$$\text{Total leukocyte count} = \text{Total of leukocytes} \times \frac{20}{0,4}$$

Percentage of leukocytes

On the 8th day, blood smear was made on the object glass and left to dry. Methanol was dropped on the dried blood smear and left to dry for 5 minutes. Giemsa stain was added and left to dry for 20 minutes. The blood smear was then rinsed with distilled water and left to dry. Immersion oil was added and examined the smear under the microscope. Count the number of eosinophils, segmented neutrophils, banded neutrophils, lymphocytes, and monocytes at 1000x magnification[9].

Macrophages phagocytic activity and capacity test

The mice were acclimatized for 7 days then administered with *Hydrocotyle sibthorpioides* extract suspension for 7 days. On the 8th day, blood sample was collected and the animals were administered with 0.5 ml *Staphylococcus aureus* R. suspension intraperitoneally. After 1 hour, the animals were sacrificed and peritoneal fluid sample was collected with a micropipette. The peritoneal fluid was smeared on the object glass, fixated with absolute methanol for 5 minutes, stained with Giemsa, left for 20 minutes, rinsed with distilled water, and left to dry. Immersion oil was added before examining the smear under the microscope with 1000x magnification. Macrophages phagocytic activity was determined based on the percentage of phagocyte that carried out phagocytosis out of 100 phagocytes. Macrophages phagocytic capacity was determined based on the number of phagocyted *Staphylococcus aureus* R by 50 active phagocytes.[10, 11]

$$\%\ \text{Macrophages activity} = \frac{\text{number of active macrophages}}{\text{total macrophages}} \times 100\%$$

Data analysis

The macrophages phagocytic activity and capacity, total leukocyte count, and percentage of leukocytes were analyzed statistically by one-way ANOVA and continued with Duncan's Multiple Range Test.

RESULTS AND DISCUSSIONS

The herb that was used in this study is *pegagan embun*. The herb was identified as *Hydrocotyle sibthorpioides* Lam, comes from a family of Araliaceae Lam. by *Anda* Herbarium, Department of Biology of Faculty of Mathematics and Natural Sciences of Andalas University.

116,126 g of extract was obtained from 1 kg of dried *pegagan embun* with an extraction yield of 11,61%. The extraction yield was in accordance with specification stated by the Herbal Pharmacopeia (2008). The organoleptic test results show that *pegagan embun* extract has thick blackish green appearance, specific odour, and bitter taste. Phytochemical screening tests show that the ethanol extract contains alkaloid, flavonoid, phenolic, and saponin compounds.

Thin layer chromatography (TLC) profile is a qualitative test to identify the marker compound (quercetin) in *pegagan embun* extract. The stationary phase used was TLC Silica gel 60 F_{254} Aluminum TLC plate. The mobile phase used were n-hexane and ethyl acetate with a ratio of 6:4. The TLC profile was examined under UV light with a wavelength of 254 nm. The retention factor (Rf) of the extract and pure quercetin was 0,51. This show that the extract contains quercetin because it has the same Rf as pure quercetin, which can be seen in Figure 1.

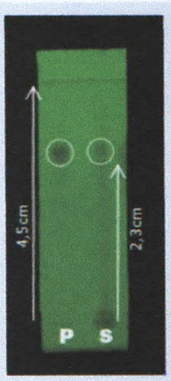

Figure 1: Thin layer chromatography profile of *pegagan embun* ethanol extract. (S) *pegagan embun* extract, (P) quercetin.

The results for macrophages phagocytic activity in animals given doses of 10, 50, 200 mg/kgbw of extract, and 50 mg/kgbw of stimuno were shown in Table 1 and Figure 2. Groups that were administered with *pegagan embun* extract show higher macrophages phagocytic activity compared to group that was administered with Na CMC 0,5%. One-way ANOVA test shows that *pegagan embun* extract influence macrophages phagocytic activity significantly ($p<0.05$). Duncan's multiple range test show that macrophages phagocytic activity increases with increasing doses ($p<0.05$). Group that was administered 200 mg/kgbw *pegagan embun* extract show the same macrophages phagocytic activity as group administered with stimuno ($p>0.05$).

The results for macrophages phagocytic capacity were shown in Table 2 and Figure 3. Groups treated with *pegagan embun* extract at doses of 10, 50, and 200 mg/kgbw show significant increase in macrophages phagocytic capacity ($p<0.05$). Groups treated with extract show higher

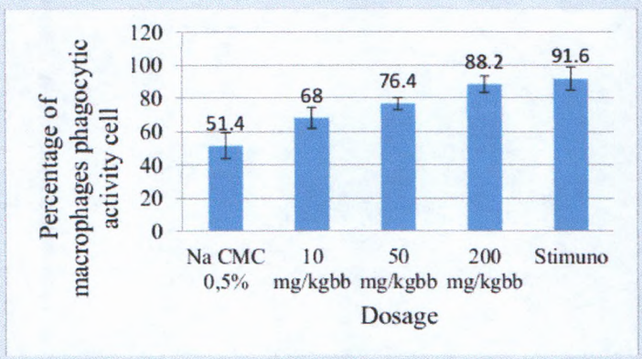

Figure 2: Bar graph of dosage versus percentage of peritoneal macrophages phagocytic activity in white male mice.

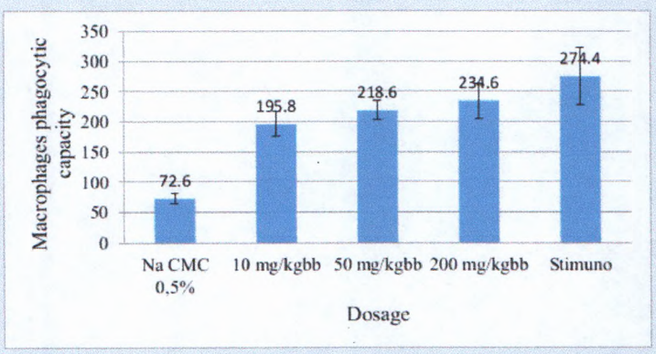

Figure 3: Bar graph of dosage versus percentage of peritoneal macrophages phagocytic capacity in white male mice.

Table 1: Percentage of peritoneal macrophages phagocytic activity in control groups and male white mice treated with *pegagan embun (Hydrocotyle sibthorpioides Lam.)* extract.

Percentage of macrophages phagocytic activity (%)	
Doses	Mean ± SD
Na CMC 0,5 %	$51,40 \pm 7,70^{a}$
10 mg/kgbw	$68,00 \pm 6,20^{b}$
50 mg/kgbw	$76,40 \pm 3,57^{c}$
200 mg/kgbw	$88,20 \pm 4,96^{d}$
Stimuno 50 mg/kgbw	$91,60 \pm 7,02^{d}$

macrophages phagocytic capacity compared to group administered with Na CMC 0.05% ($p<0.05$). There was no significant difference in macrophages phagocytic capacity between groups given extract at doses of 10 mg/kgbw and 50 mg/kgbw. Based on the previous study, flavonoid has been proven to increase IL-2 and proliferation of lymphocytes.[12] Lymphocytes proliferation will influence CD4+ cell that activates Th1 cell. Activated Th1 cell will then influence Specific Macrophage Activating Factor (SMAF) wherein SMAF are multiple molecules, for example, IFN-γ. IFN-γ will activates macrophage and increase their phagocytic activity and capacity. This results in faster rate of phagocytosis of bacteria by macrophages.[2]

The results for total leukocyte count of five tested groups were shown in Table 3 and Figure 5. Groups treated with *pegagan embun* extract at doses of 10, 50, and 200 mg/kgbw show significant increase in total leukocyte count ($p<0.05$). Groups treated with extract show higher total leukocyte count compared to group administered with Na CMC 0.05% ($p<0.05$). Group treated with 200 mg/kgbw extract show the highest total leukocyte count compared to groups treated 10 and 50 mg/kgbw extract ($p<0.05$). There was no significant difference in total leukocyte count between groups given extract at doses of 10 mg/kgbw and 50 mg/kgbw ($p>0.05$). There was no significant difference in total leukocyte count between animals given extract at doses of 200 mg/kgbw and stimuno ($p>0.05$). Based on the previous study, increase in total leukocyte count indicates increase in immune response.[13]

The results for percentage of leukocytes were shown in Table 4. One-way ANOVA test shows that *pegagan embun* extract at doses of 10, 50, and 200 mg/kgbw influence the percentage of segmented neutrophils and lymphocytes significantly ($p<0.05$) but show no significant difference towards percentages of banded neutrophils, eosinophils, and monocyes ($p>0.05$). There was no significant difference in percentages of segmented neutrophils in group treated with 10 mg/kgbw extract compared to group treated with doses of 50 mg/kgbw ($p>0.05$) and they show the same effect as 50 mg/kgbw stimuno. The percentage of segmented neutrophil decreases after the administration of *pegagan embun* extract. Hence, it can be assumed that macrophages played the most important role in phagocytosis or there was an increase in chemotaxis factor that increase the phagocytosis ability.[2]

Pegagan embun extract significantly increases the percentage of leukocytes as shown in Figure 6. Groups treated with extract at doses of 10 and 50 mg/kgbw show no significant difference in percentage of leukocytes compared to control group ($p>0.05$). Group given extract at doses of 200 mg/kgbw shows higher percentage of leukocytes compared to group given extract at doses of 50 mg/kgbw. There was no significant difference on the percentage of leukocytes in group treated with extract at doses of 200 mg/kgbw compared to group treated with stimuno at doses of 50 mg/kgbw. Increase in percentage of lymphocytes indicates that *pegagan embun* extract could stimulate specific immune response.[2]

This study show that *pegagan embun* (*Hydrocotyle sibthorpioides* Lam.) extract at doses of 10, 50, and 200 mg/kgbw show immunostimulant properties through increasing the activity and capacity of macrophages, total leukocyte count, percentage of lymphocytes, and decreasing the number of segmented neutrophils. Extract at doses of 50 and 200 mg/kgbw show simillar immunostimulatory effect.

Table 2: Mean of peritoneal macrophages phagocytic capacity in control groups and male white mice treated with *pegagan embun (Hydrocotyle sibthorpioides Lam.)* extract.

Mean of peritoneal macrophages phagocytic capacity	
Doses	**Mean ± SD**
Na CMC 0,5 %	72,60 ± 8,08[a]
10 mg/kgbw	195,80 ± 20,93[b]
50 mg/kgbw	218,60 ± 15,75[bc]
200 mg/kgbw	234,60 ± 28,91[c]
Stimuno	274,40 ± 46,77[d]

Table 3: Mean of total leukocyte count in control groups and male white mice treated with *pegagan embun (Hydrocotyle sibthorpioides Lam.)* extract.

Total leukocyte (/µL blood)	
Doses	**Mean ± SD**
Na CMC 0,5 %	4370 ± 496,99[a]
10 mg/kgbb	8540 ± 482,70[b]
50 mg/kgbb	8810 ± 207,36[b]
200 mg/kgbb	9460 ± 403,73[b]
Stimuno	10020 ± 668,58[c]

Table 4: Percentage of leukocytes in control groups and male white mice treated with *pegagan embun (Hydrocotyle sibthorpioides Lam.)* extract.

Doses	Percentage of leukocytes (%) Mean ± SD				
	Segmented neutrophils	Banded neutrophils	Eosinophils	Lymphocytes	Monocytes
Na CMC 0,5 %	45,40 ± 3,36[d]	3,60 ± 1,51	1,80 ± 0,83	45,80 ± 2,16[a]	3,40 ± 2,30
10 mg/kgbw	41,20 ± 1,92[c]	4,40 ± 1,51	2,20 ± 0,83	47,80 ± 3,11[ab]	4,40 ± 1,34
50 mg/kgbw	40,00 ± 1,58[bc]	3,60 ± 1,51	2,20 ± 0,83	50,00 ± 2,23[b]	4,20 ± 0,83
200 mg/kgbw	35,20 ± 1,64[a]	4,00 ± 1,58	2,20 ± 1,30	53,80 ± 2,28[c]	4,80 ± 2,04
Stimuno	37,60 ± 2,50[ab]	1,80 ± 0,83	1,80 ± 0,83	55,60 ± 1,14[c]	3,20 ± 1,09

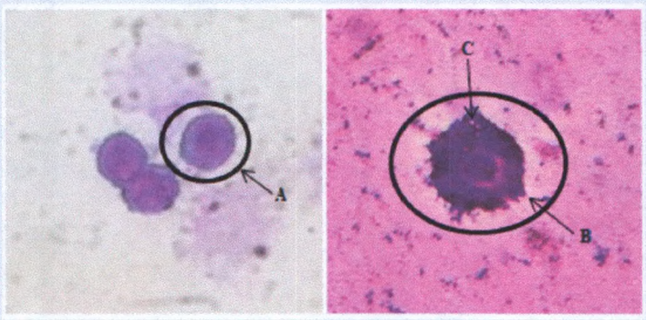

Figure 4: Peritoneal macrophages of white male mice. (A) macrophage, (B), activated macrophage, (C) *Staphylococcus aureus*.

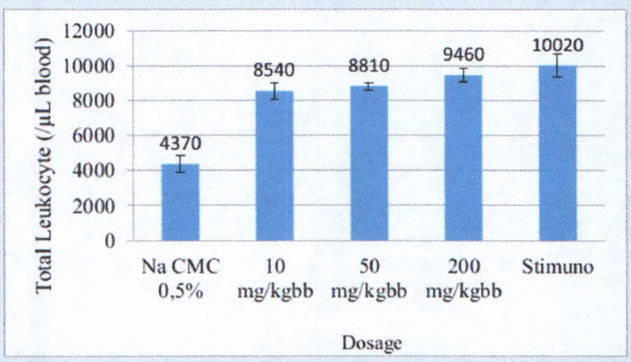

Figure 5: Bar graph of dosage versus total leukocyte count in white male mice.

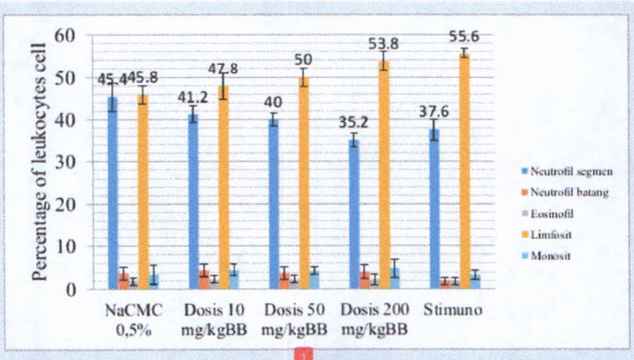

Figure 6: Bar graph of dosage versus percentage of leukocytes in white male mice.

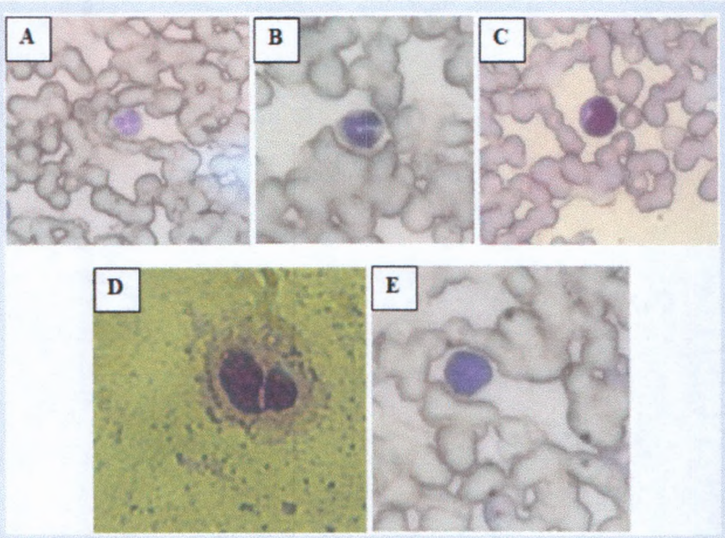

Figure 7: White male mice leukocytes. (A) Segmented neutrophil, (B) Banded neutrophils, (C) Monocyte, (D) Eosinophil, (E) Lymphocyte.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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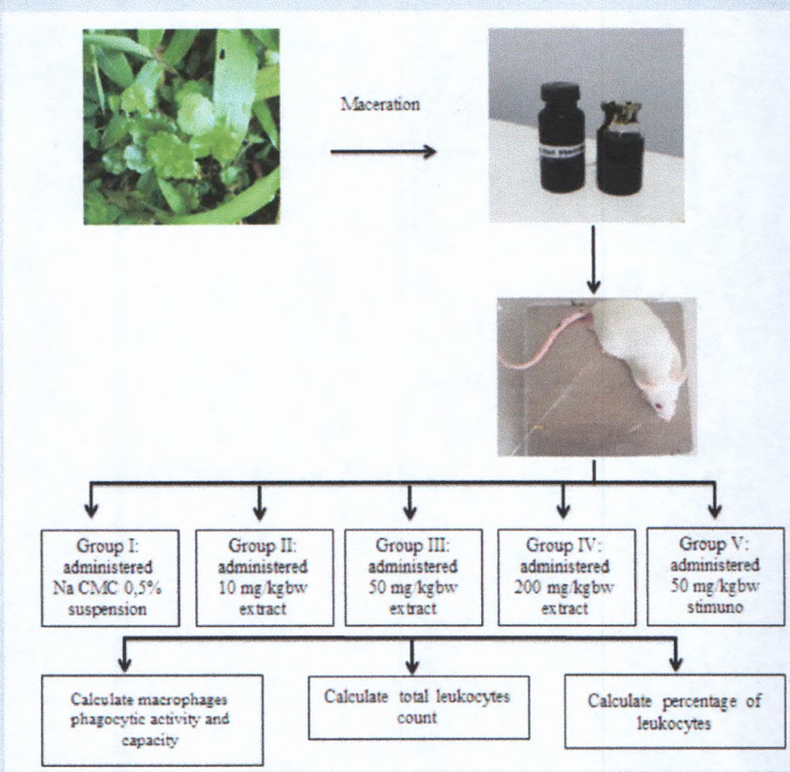
GRAPHICAL ABSTRACT



SUMMARY

Pegagan embun (Hydrocotyle sibthorpioides L.) is known for its immunostimulatory effect. The aim of this research is to study the effect of *pegagan embun* ethanol extract on macrophages phagocytic activity and capacity, total leukocyte count, and percentage of leukocytes. 25 male white mice were divided into 5 equal groups. Negative control group were given Na CMC 0.5%, the extract treated groups were given ethanol extract of *pegagan embun* herbs at doses of 10, 50, 200 mg/kgbw, and the positive control group was given Stimuno 50 mg/kgbw orally for 7 days. On the 8th day, the total and percentage of leukocytes were counted through the blood sample taken intravenously. The mice were then induced with *Staphylococcus aureus* suspension. The results shows significant increase of concentration ($p<0.05$) towards macrophages phagocytic activity and capacity, and total leukocytes count. Percentage of leukocytes show that lymphocytes increases significantly ($p<0.05$), meanwhile neutrophils segments decreases significantly ($p<0.05$). It can be concluded that ethanol extract of *pegagan embun* herb at doses of 10, 50, 200 mg/kgbw shows immunostimulatory activity through increasing macrophages phagocytic activity and capacity, increasing the number of lymphocytes, and decreasing the number of segmented neutrophils. *Pegagan embun* extract at doses of 200 mg/kgbw show simillar immunostimulatory effect as 50 mg/kgbw stimuno.

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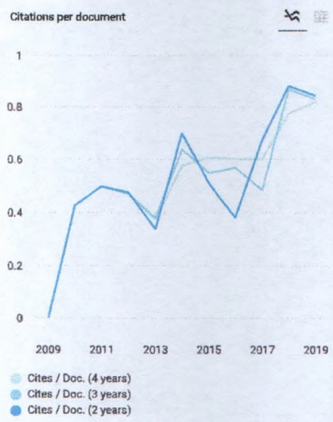

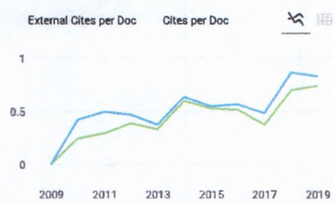

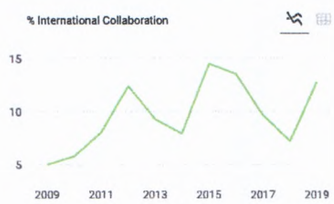

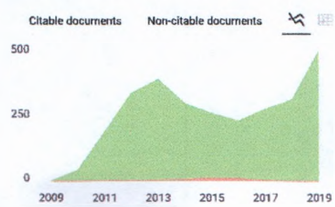

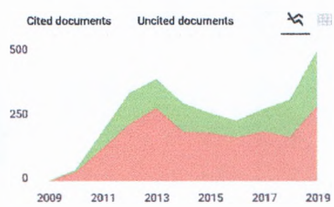

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