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Original Article

IMMUNOSTIMULANT ACTIVITY OF HANTAP (*STERCULIA COCCINEA* JACK) LEAVES EXTRACT ON NON-SPECIFIC AND SPESIFIC IMMUNE RESPONSES

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ABSTRACT

Objective: This study aims to determine the immunostimulant effect and the effective dose of hantap leaf ethanolic extract against non-specific and specific immune responses.

Methods: The immunostimulant activity of the extract was tested by the carbon clearance method (non-specific response). The effect of the extract on delay-type hypersensitivity/DTH (cellular-specific response) was determined by the paw edema method. The number of leukocyte cells and the percentage of leukocyte cell types were also calculated. The antibody titer (humoral-specific response) test was carried out by the hemagglutination method.

Results: The results of the non-specific immune response demonstrated that three doses of hantap leaf extract, when compared to the negative control, could accelerate the rate of carbon elimination and increase the phagocytosis index. Hantap leaf extract can provide an immunomodulatory effect by increasing the delayed-type hypersensitivity response by showing a greater volume of leg swelling than the negative control received CMC Na 0.5%, which was significantly different ($p<0.05$), increasing leukocyte count and leukocyte differential and increasing primary and secondary antibody titers. The effective dose of hantap leaves extract was 200 mg/kg body weight.

Conclusion: This study proves that hantap leaf extract has an immunomodulatory effect that increases the immune system (immunostimulant)

Keywords: Antibody titer, Carbon clearance, Delayed-type hypersensitivity, Immunostimulant, Leukocytes

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INTRODUCTION

The immune system plays a crucial function in maintaining physiological and immunological health. Diseases such as rheumatoid arthritis, cancer, and other inflammatory disorders can develop due to impaired immune system function [1, 2]. The immune system comprises white blood cells, including lymphocytes, monocytes, neutrophils, and macrophages, as well as specific immunological components such as antibodies, proteins, and cytokines. This defence mechanism safeguards the body against various infections and disorders [3]. Maintaining homeostasis in the immune system is a protective mechanism against the onset of several illnesses. An imbalance in equilibrium can give rise to immune-mediated ailments, including infection, inflammation, metabolic syndrome, and cancer [4-6].

The human body exhibits immunological responses that can be categorized into two distinct types: innate immunity and adaptive immunity. The natural immune system, or non-specific immunity, is crucial in the initial defense against various pathogens. On the other hand, adaptive immunity, or acquired or specific immunity, develops gradually and functions as a targeted defense mechanism against infections [7, 8].

The immune system plays an important role in the pathogenesis of disease. Almost all diseases are related to the immune system, including infections, inflammation, autoimmune diseases, and malignancies. This indicates that the immune system can change, and at the same time, it can modulated in various ways, namely by using immunomodulators. Therefore, currently, immunomodulators have a quite prospective position in treating a disease. Immunomodulators comprise synthetic, biological, and natural substances that possess the capacity to regulate the immunological response of both the innate and adaptive immune systems. Immunomodulators are utilized in the therapeutic management of conditions that affect the normal functioning of the immune system [9]. Immunomodulators are additionally employed to rectify immune responses that are characterized by imbalances. Numerous synthetic immunomodulatory drugs have been discovered; however, their clinical efficacy has been hindered by challenges related to absorption, stability, and significant adverse effects [10]. Therefore, it is essential to create novel immunomodulators with minimal to no adverse effects to treat immune-related disorders. The utilization of natural plants and their secondary metabolites plays a crucial role in managing immunological illnesses through the modulation of immune responses. The varied pharmacological effects can be related to its immunomodulatory and antioxidant capabilities [11].

Hantap (*Sterculia coccinea* Jack) plant is one of the local wisdom plants and is used as traditional medicine in Central Sulawesi for breast, brain, leukemia, uterine, cervical, and prostate cancers [12]. Hantap leaf extract has antioxidant activity in the category of strong to very strong with the 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC) methods. There is a close relationship between antioxidants and the immune system. Many studies have proven that antioxidants can stimulate and protect the cellular immune system. Antioxidants can increase macrophage activity, protect macrophages from autooxidation damage, and increase lymphocyte proliferative responses [13]. Numerous investigations have provided evidence for the presence of secondary metabolites, including tannins, alkaloids, flavonoids (specifically 5,7-dihydroxy-3-(4'-hydroxy benzyl) chromone and kaempferide-3-O-α-L-rhamnosyl-7-O-α-L-rhamnoside), saponins, steroids, and triterpenoids. These compounds have demonstrated promising immunomodulatory properties [14-16]. More scientific data is needed to support the immunomodulatory properties of hantap leaf extract. Therefore, this study conducted an experiment to demonstrate hantap leaf extract's immunomodulatory efficacy against non-specific and specific immunological responses.

MATERIALS AND METHODS

Materials

Hantap leaves are collected directly from plants in Selena Village, Buluri-Ulujadi, Central Sulawesi. The plant was identified as *Sterculia coccinea* Jack by the Laboratory of Plant Biosystematics,

Faculty of Mathematics and Natural Sciences, Tadulako University No. 589/UN 28.1.28/BIO/2022. The chemicals used in this research are as follows: 96% ethanol, nutrient agar media (NA), nutrient broth (NB), CMC Na, Waterone, physiological NaCl, Levamisole (Askamex®, PT. Konimex), Stimuno® tablets (PT. Dexa Medica), bovine red blood cells (BRBC) 2%, Pelican China Ink B-17®, phosphate buffer saline (PBS), Ketamin-Hameln® (10 mg/ml), *Staphylococcus aureus* ATCC 25293.

Extract preparation

Hantap leaf simplicia was extracted using the maceration technique utilizing 96% ethanol as the solvent. The extraction process was conducted for 5 d. The gathered macerate was evaporated using a rotary evaporator at 60 °C. This was followed by further evaporation in a water bath until a thick extract was obtained. Next, the percentage yield is determined.

Ethical approval

The research methods were conducted according to the ethical approval of the Medical and Health Research Ethics Committee of the Faculty of Medicine at Tadulako University No. 8780/UN 28.1.30/KL/2022.

Non-specific immune responses with a carbon clearance test

The experimental subjects were male Balb/C mice aged between 2 and 3 mo. The mice used in the experiment were divided into five groups. Group 1 received a suspension of 0.5% sodium carboxymethyl cellulose (Na CMC) as a negative control. Group 2 was administered Stimuno® at a dose of 19.5 mg/kg BW as the positive control. Meanwhile, groups 3, 4, and 5 were given Hantap leaf extract (HLE) suspensions at 50, 100, and 200 mg/kg BW doses, respectively. The phagocytic activity of each group was assessed using the carbon clearance method, with therapy administered for seven consecutive days [17]. Blood samples were taken from each group on the eighth day of the experiment. The blood collected in blood collection tubes containing sodium citrate. A volume of 25 microliters of blood was collected, and a solution containing 1% acetic acid was introduced to initiate the red blood cell lysis process. The baseline blood sample was used as blank (0 min). Next, the tail vein was intravenously administered carbon suspension (0.1 ml/10 g BW). Blood samples were collected 5 and 20 min after injection and placed in a Na-citrate tube. 25 µl from each blood sample mixed with 4 ml of 1% acetic acid to induce red blood cell lysis. Absorption measurements were carried out with a UV-visible spectrophotometer at 632.0 nm. After 12 h, the blood was taken, the mice were sacrificed, the liver and spleen of the mice were taken and weighed, and the weight of the liver and spleen was recorded. Calculated carbon elimination rate constant (K) and phagocytosis index using the formula [18, 19]:

$$\text{Carbon elimination rate constant (K)} = \frac{\text{Log OD5} - \text{Log OD20}}{\text{T2} - \text{T1}} \quad \ldots\ldots\ldots\ldots (1)$$

$$\text{Phagocytosis index } (\alpha) = \frac{K^{1/3} \text{x Animal Height}}{\text{Liver weight} + \text{Lymph weight}} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Specific immune response activity with an antibody titer test

A specific immune response test was carried out by determining primary and secondary antibody titers using hemagglutination methods and test delayed-type hypersensitivity as a cellular immune response. The antigen in specific immune response tests was 2% bovine red blood cells in PBS. Each group of experimental animals was injected with 2% BRBC in PBS as an intraperitoneal antigen on day 1. Rats from groups 1, 2, 3, 4, and 5 were given test preparations orally daily for 11 d. Group 1 received a suspension of 0.5% Na CMC as a negative control in this study. Group 2 was administered a suspension of Levamisole at a dose of 13.5 mg/kg BW as the positive control. Meanwhile, groups 3, 4, and 5 were administered a suspension of hantap leaf extract (HLE) at 50, 100, and 200 mg/kg BW, respectively. On the 6th day, rats blood was taken through the tail vein and centrifuged to obtain serum. The hemagglutination value on day seven was expressed as the primary antibody titer. On the 12th day, the rats were again taken for blood to obtain serum, and the hemagglutination technique was again performed to determine the antibody titer. This hemagglutination value was expressed as the secondary antibody titer. The blood sample was collected in a microtube, the blood was allowed to clot for 1-2 h, then centrifuged at 3000 rpm for 10 min, and the serum was taken. The serum was diluted with a double dilution of 1/4 to 1/512. The double dilution method was carried out as follows: first, 25 µl of serum was added with 75 µl of PBS pH 7.4 and homogenized until the lowest dilution was 1/4. Then 50 µl of the 1/4 dilution was added with 50 µl of PBS pH 7.4 and homogenized until a 1/8 dilution was obtained. Next, 50 µl of the 1/8 dilution was added with 50 µl of PBS pH 7.4 and homogenized until a 1/16 dilution was obtained. The same procedure was carried out for each dilution until the highest dilution was 1/512 and the volume of each dilution was 50 µl. Then 50 µl of 2% BRBC were added to each well. Then incubated at 37 °C for 1 hour and visually observed for hemagglutination. The antibody titer value was determined based on the final dilution at which the antibody remained detectable through visually observable hemagglutination. This hemagglutination manifested as distinct dark red clumps directly apparent during incubation. The antibody titer result is subsequently subjected to a mathematical transformation, specifically the application of the function [2log(titer)+1] [20].

Specific immune responses with a delayed-type hypersensitivity (DTH) test

The specific cellular response assessment used delayed-type hypersensitivity. The delayed-type hypersensitivity (DTH) response activity of the extract was investigated by the increase or decrease in paw volume based on the modified method by Ahirwal *et al.* (2015) [21]. Wistar rats were divided into five groups. Each group was further divided into a negative control group (administered with Na CMC 0.5%), a positive control (orally administered with levamisole 13 mg/kg BW), and a treatment group (orally administered with Hantap leaf extract at various doses of 50, 100, and 200 mg/kg BW. Given appropriate treatment by group division orally every day for 14 d. Injected on the 4th day of bacterial suspension *Staphylococcus aureus* as much as 0.1 ml intraperitoneally in all groups. On the 14th day, measurements were taken of the volume of rat feet that had previously been marked with a limit volume measurement using markers. The foot volume of the rat was measured as an initial volume (V0). Re-injected intraplantar 0.1 ml suspension of *S. aureus* bacteria. On day 15 (after 24 h), the volume of rat feet was measured by a digital plethysmometer. Measurements are made by dipping the rat's foot into the plethysmometer, and the increase in scale on the plethysmometer indicates the specific time volume (Vt) of rat the feet. The foot thickness in each test group was measured before and after the induction of *S. aureus*, and the difference in thickness was calculated as a percentage. Blood was collected on day 15, and the serum was used for the determination of total leukocytes and differentia leukocytes.

Statistical analysis

The data were presented as the mean±standard deviation (SD) and were analyzed using SPSS version 26.0. The data analysis employed a one-way ANOVA, followed by Duncan's test at a confidence level of 95%.

RESULTS AND DISCUSSION

Preparation of hantap leaf extract (HLE)

The results of maceration of the dry powder of hantap leaves obtained a thick extract with ina yield of 15.7%. Based on the study, Hantap leaf extract also detected to contain alkaloids, flavonoids, saponins, tannins, and steroids.

Non-specific immune responses with a carbon clearance test

Table 1 displayed the data on the carbon particle removal rate across all experimental group. An immunomodulator test using the carbon clearance carbon method was done to measure the activity of non-specific immune cells (phagocytic cells) to eliminate pathogenic cells that enter the body. This test is based on the speed at which phagocytic cells ingest. The foreign substance after being administered the immunomodulatory substance. The foreign substances or particles used in this experiment were carbon ink.

Carbon enters the body recognized as nonself and will be phagocytosed by macrophages, neutrophils, monocytes, and other leukocytes, so the amount in the blood will decrease with increasing time The carbon clearance method can be employed to evaluate the phagocytic activity assay, which provides insight into the functionality of the non-specific immune system in phagocytosis against foreign particles within the bloodstream [22].

The carbon clearance technique assesses the efficacy of phagocytic cells in eliminating harmful microorganisms that invade the organism. The assessment of immune function often involves the utilization of phagocytosis as a prominent immunological parameter. The carbon elimination rate, phagocytosis constant and the phagocytosis index are the parameters used to assess immunomodulatory activity. A high phagocytosis constant value indicates immunostimulatory activity, which means that the the phagocytic cells carry out the phagocytosis process faster [23]. Table 1 showed that the carbon elimination constant values and phagocytosis index of the HLE group at doses of 50, 100, and 200 mg/kg BW and the positive control (Stimuno 19.5 g/kg BW) were greater than the negative control. The greater the phagocytosis index, the higher the carbon clearance speed. The increase in the phagocytosis index value at various doses of HLE indicated immunostimulatory activity. The higher the dose of hantap leaf extract, the greater the immunostimulating effect.

The quantification of the capacity or efficacy of phagocytosis in removing carbon particles is represented as a phagocytosis index. An observed elevation in the carbon clearance index signifies an enhancement in the phagocytic capability of mononuclear macrophages, hence indicating an improvement in non-specific immunity [24].

Table 1: Phagocytic activity in the carbon clearance test

Group	Carbon elimination rate constant (mean±SD)	Phagocytosis index	Classification of immunomodulating effects
Negative control	0.0015273±0.0009[a]	1.00	-
Stimuno 19.5 g/kg BW	0.0113999±0.0030[b]	3.57	Immunostimulant
HLE 50 mg/kg BW	0.0044971±0.0040[a]	2.62	Immunostimulant
HLE 100 mg/kg BW	0.0049110±0.0032[a]	3.28	Immunostimulant
HLE 200 mg/kg BW	0.0105761±0.0020[b]	3.58	Immunostimulant

*Data expressed as mean±SD; HLE = ethanol extract of hantap leaves; different alphabets indicate a significant difference ($p<0.05$). n=5

Based on table 1, the phagocytosis index in each test group increased compared to the negative control group. It was also seen that there was a relationship between the dose of HLE and the phagocytic index; that is, the greater the dose of HLE, the higher the phagocytic index. This finding shows that Hantap leaf extract has the potential to induce an immunostimulatory response. The positive control employed in this study was Stimuno®, a product that comprises Meniran extract (*Phyllanthus niruri*). Previous preclinical and clinical investigations have demonstrated the immunostimulant properties of this extract. The relationship between the phagocytosis index and the phagocytosis constant is one of direct proportionality. This implies that as the value of the phagocytosis constant and index increases, the rate of the phagocytosis process performed by phagocytic cells in removing carbon from the bloodstream will also increase. A higher phagocytic index indicates increased monocyte-derived macrophage phagocytosis within the non-specific immune system. The phagocytic index is determined by conducting a comparative analysis between the experimental and control groups. According to Wagner (1989), a phagocytic index of less than 1 is an immunosuppressive, if between>1 and 1.5 indicates a moderate immunostimulating effect and a value of more than 1.5 indicates a strong immunostimulating effect [25].

Specific immune responses activity with an antibody titer test

Primary antibody titers indicate stimulation of B lymphocytes, T lymphocytes, and macrophages, while secondary antibody titers indicate stimulation of B cells memory in the process of antibody formation. The observation of antibody titers was carried out by looking at the hemagglutination of the highest dilution of rat blood serum, which still gave a positive hemagglutination reaction [26]. As shown in table 2, HLE works well on both primary and secondary antibody titers based on the response to the hemagglutination method. In this study, levamisole was used as a positive control because it boots the immune system by stimulating antibody formation and enhancing T-cell response by activating and proliferating T cells [27].

Table 2: Effect of different doses of HLE on primary and secondary antibody titers

Group	Primary antibody titer value	Secondary antibody titer value
Negative control	4.90±0.17[a]	4.90±0.18[a]
Levamisole 13.5 mg/kg BW	5.95±0.28[b]	5.95±0.29[b]
HLE 50 mg/kg BW	5.80±0.34[b]	5.50±0.39[ab]
HLE 100 mg/kg BW	6.05±0.14[b]	5.80±0.24[b]
HLE 200 mg/kg BW	6.25±0.15[b]	6.25±0.15[b]

*Data expressed as mean±SD; HLE = ethanol extract of Hantap leaves; Different alphabets indicate a significant difference ($p<0.05$). n=5

Increasing the HLE dose showed an increase in hemagglutination values, although not significantly different statistically. It was concluded that the larger the dose given, the more antibodies there would be, so that the bond between the antigen and the antibodies formed would be greater. The antibody titer test showed the activity of the humoral immune response, which involves the interaction process between B lymphocytes and the 2% BRBC antigen, namely the proliferation and differentiation of B lymphocytes into plasma cells that secrete primary and secondary antibodies. Antibodies act as effector humoral responses by opsonizing antigens, resulting in the neutralization of the antigen and facilitating the elimination of the antigen involved so that it is more easily phagocytosed by macrophages [28]. The primary antibody titer results in the HLE group at doses of 50, 100, and 150 mg/kg BW were higher than those in the negative control group, and their activity was not significantly different from the levamisole group. An increase in primary antibody titers indicates the stimulation of B lymphocytes (secreting IgM antibodies). Meanwhile, there was also an increase in secondary antibody titers, which indicated stimulation of memory B cells in the formation of IgG antibodies [29]. The results showed that hantap leaf extract increased the antibody titer value of male white rats' immune cells. Increased activity of Th2 cells can stimulate production and can increase B cell activity in the formation of antibodies. Therefore, hantap leaf extract can be used as an immunostimulator because of its effect on increasing the antibody titer of rat immune cells.

Specific immune responses with a delayed-type hypersensitivity (DTH) test

In testing the delayed-type hypersensitivity response, the injected *S. aureus* bacteria acts as an antigen, which can cause edema due to the inflammatory response (accumulation of macrophages, leukocytes, and other immune cells) so that within 24 h the edema will be visible [30]. The first antigen induction (day 4) intraperitoneally is called the sensitization phase. The bacteria penetrate the skin and form a complex with epidermal carrier proteins to form antigens and attach to the surface of the APC (Antigen-Precenting Cell). APC will activate helper cells, especially Th1 cells. The introduction of antigen on day four will trigger the formation of memory cells to remember the same antigen when exposed to it a second time. The second intraplantar administration of antigen (day 14) is called the effector phase. APC presents the antigen and will be recognized by T cells, which will induce a reaction. The inflammatory reaction is mediated by specific cellular components of the immune system and is characterized by the release of inflammatory mediators, which will trigger macrophages to release cytokines.

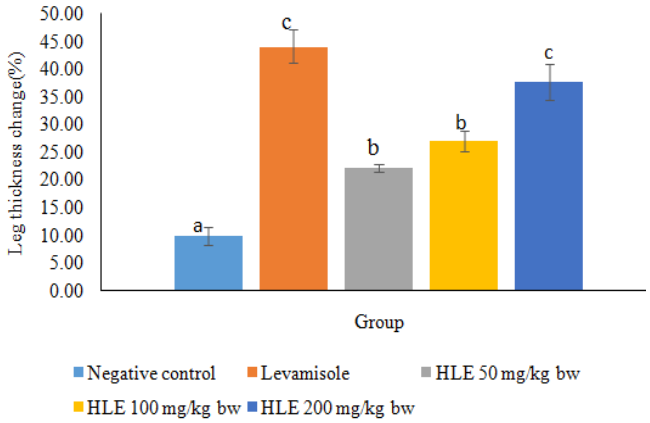

Fig. 1: Effect of different doses of HLE on rat paw volume infected with *S. aureus,* *Data expressed as mean±SD; HLE = ethanol extract of hantap leaves; different alphabets indicate a significant difference (p<0.05); n=5

Data on specific cellular immune responses determined through the DTH test can be seen in fig. 1. In this study, Staphylococcus aureus bacteria were used as an antigen or inducer, resulting in the formation of Th cells, which help increase active B cells into plasma cells, strengthen active cytotoxic T cells, suppress T cells, and activate macrophages. The cells can act as lymphocytes, releasing pro-inflammatory (swelling) cytokines. Antigens in lipopolysaccharide cause macrophages to release TNF (tumor necrosis factor) and IL-1, which are inflammatory mediators. Swelling in T24 is caused by increased macrophage activation during cytokine production, causing the accumulation of cytokines (IL-12) at the induction site. This indicates a slowly progressive hypersensitivity reaction. Based on the theory of delayed type hypersensitivity (type IV), leg volume increases within 6-12 h and reaches maximum intensity after 24-72 h. The delay in response time reflects the time required for cytokines to induce macrophage activation [31].

The immunomodulatory effect test on delayed-type hypersensitivity responses was carried out by providing antigen induction to test animals, which will influence and activate macrophage cells so that a non-specific immune response will occur. If non-specific immunity, which is influenced by macrophage cells, works, a specific immune response will occur, which involves the activation of Th cells, which will release inflammatory cytokines and increase macrophage activity. One response to the emergence of delayed-type hypersensitivity is the release of inflammatory mediators that increase the activity of macrophage cells, such as IL-1, IL-2, and IL-4 receptor antagonists, which is characterized by swelling in the rat paw [32].

The percentage change in the volume of the paws of mice after administration of HLE at doses of 50, 100, and 200 mg/kg BW was higher than the negative control and showed a significant difference. This shows that HLE can increase the volume of the test mice's paws through a response to $CD^{4+}$and CD^{8+}T cells, resulting in tissue damage and inflammation. The HLE dose of 200 mg/kg is an effective dose.

In addition to delayed-type hypersensitivity, the total leukocyte count and percentage of leukocyte cells are also determined after administering HLE. The administration of HLE can increase total leukocytes, as shown in fig. 2. The group given HLE at doses of 50, 100, and 200 mg/kg BW showed a significant difference ($p<0.05$) compared to the negative control (Na CMC 0.5%). HLE at 200 mg/kg BW showed the highest total leukocytes compared to 50 and 100 mg/kg BW doses and no significant differences with levamisole.

Based on the results of the differential leukocyte measurements in fig. 3, it can be seen that the group given 0.5% Na-CMC (negative control) had lower numbers of eosinophils, basophils, monocytes, neutrophils, and lymphocytes and was significantly different from the positive control and the HLE group. Data differential leucocytes, especially lymphocytes, show that an increase in the number of lymphocytes can increase the number of lymphocyte cells, which are immune cells that play an important role in humoral and cellular response systems. Although the HLE doses of 50 and 100 mg/kg BW showed no significant difference compared to the negative control, there was an increase in the number of differential leukocyte profiles, especially at the HLE dose of 200 mg/kg BW.

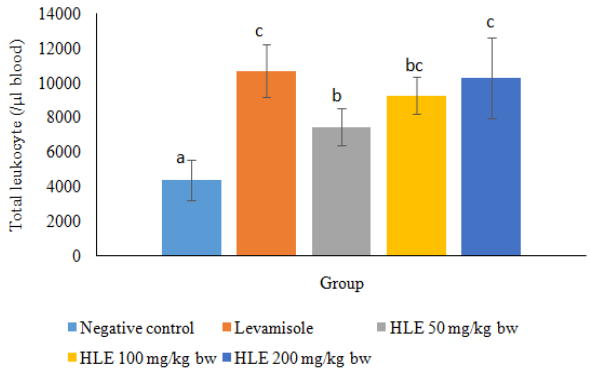

Fig. 2: Effect of different doses of HLE on total leukocytes, *Data expressed as mean±SD; HLE = ethanol extract of hantap leaves; different alphabets indicate a significant difference ($p<0.05$); n=5

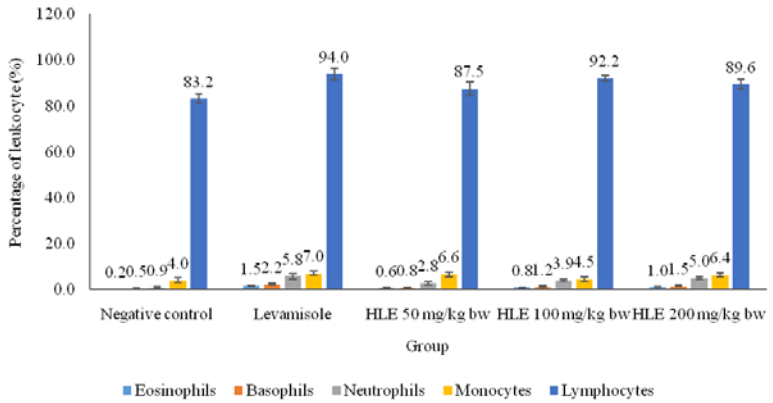

Fig. 3: Effect of different doses of HLE on leukocytes differential profile, *Data expressed as mean±SD; HLE = ethanol extract of Hantap leaves; different alphabets indicate a significant difference ($p<0.05$); n=5

Increased levels in the leukocyte differential profile indicate an immunostimulatory effect [33] on HLE. This is due to secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and steroids, according to the results of the phytochemical screening.

Flavonoids are one of the compounds that can boost the immune system. Compounds that play a role in the immune response of test animals are flavonoids. Flavonoids can significantly increase leukocytes and humoral responses by stimulating a subset of macrophages and B-lymphocytes which play a role in antibody synthesis. Flavonoids can also be expressed as immunomodulators that can enhance the immune system. At the same time, saponins will act as immunosuppressors (substances that suppress or decrease the immune system). Saponins produce cytokines such as interleukins and interferons, which play a role in immunostimulant effects. Interleukins and interferons will react with antigens that enter the body. Saponins act as immunostimulators. Tannin compounds can also affect human physiological activities, such as stimulating phagocytic, antitumor, and antibacterial cells. Alkaloids increase the immune response by increasing the activity of IL-2 (interleukin 2) and lymphocyte proliferation. Steroids can act as an immunostimulator that stimulates the immune system by increasing its defense against bacterial attacks by inducing the multiplication of white blood cells. Steroids activate cellular defense cells by increasing cells that act as immune macrophages, granulocytes, and T and B lymphocytes [34-36]. The results of the research show that Hantap leaf extract has the potential to be developed into a powerful immunomodulator for modulating the immune response. However, the mechanism of immunostimulation at a molecular level still needs to be investigated, including quantitative measurement of the levels of antibodies formed.

CONCLUSION

Hantap leaf extract exhibits immunomodulatory properties through its ability to enhance the immune system, acting as an immunostimulant. An effective dose is achieved at 200 mg/kg BW.

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ETHICAL APPROVALS

The research process adhered to the ethical approval of the Ethics Committee for Medical and Health Research at the Faculty of Medicine, Tadulako University.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally. The authors collectively participated in the drafting and critically revising the article, ensuring the inclusion of significant intellectual material. They agreed to submit the report to the present journal and provided final consent for publication. Furthermore, the authors decided to assume responsibility for all elements of the work.

CONFLICT OF INTERESTS

All authors assert that they do not possess any conflict of interest.

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