Immune responses that are characterized by imbalances. Numerous crucial in the initial defense against various pathogens. On the other immunity. The natural immune system, or non-specific immunity, is categorized into two distinct types: innate immunity and adaptive 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-alpha-L-rhamnosyl-7-O-alpha-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, Bulur-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. S89/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 (Askamek®, PT. Konimes), Stimuno® tablets (PT. Dexa Medica), bovine red blood cells (BRBC), 9%, 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% 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-citra tube. 25 µl from each sample mixed with 4 µl 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]:

\[ \text{Carbon elimination rate constant (K)} = \frac{\log_{2} \text{OD}_{5}}{\log_{2} \text{OD}_{20}} \]  
\[ \text{Phagocytosis index (x)} = \frac{\text{Liver weight} \times \text{Lymph weight}}{\text{Animal Height} \times \text{Liver weight} + \text{Lymph weight}} \]  

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 hemagglutination specific immune response test 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 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 tube 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 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 sample procedure was carried out until the lowest 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 h and visually observed for hemagglutination. The antibody titer value was determined based on the final dilution at which the antibody remained detectable through visibly 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(抗体倍数)]= [20].

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 non-specific immune cells (phagocytic cells) to eliminate pathogenic substances or particles used in this experiment were carbon ink.

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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 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].

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 give 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 boosts the immune system by stimulating antibody formation and enhancing T-cell response by activating and proliferating T cells [27].

### Table 1: Phagocytic activity in the carbon clearance test

<table>
<thead>
<tr>
<th>Group</th>
<th>Carbon elimination rate constant (mean±SD)</th>
<th>Phagocytosis index</th>
<th>Classification of immunomodulating effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.0033±0.0020</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>Stimuno 19.5 g/kg BW</td>
<td>0.0133±0.0020</td>
<td>3.57</td>
<td>Immunostimulant</td>
</tr>
<tr>
<td>HLE 50 mg/kg BW</td>
<td>0.0044±0.0020</td>
<td>2.62</td>
<td>Immunostimulant</td>
</tr>
<tr>
<td>HLE 100 mg/kg BW</td>
<td>0.0049±0.0020</td>
<td>3.28</td>
<td>Immunostimulant</td>
</tr>
<tr>
<td>HLE 200 mg/kg BW</td>
<td>0.0167±0.0020</td>
<td>3.58</td>
<td>Immunostimulant</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary antibody titer value</th>
<th>Secondary antibody titer value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>4.90±0.17±</td>
<td>4.90±0.18±</td>
</tr>
<tr>
<td>Levamisole 13.5 mg/kg BW</td>
<td>5.95±0.28±</td>
<td>5.95±0.29±</td>
</tr>
<tr>
<td>HLE 50 mg/kg BW</td>
<td>5.30±0.34±</td>
<td>5.50±0.39±</td>
</tr>
<tr>
<td>HLE 100 mg/kg BW</td>
<td>6.05±0.14±</td>
<td>5.80±0.24±</td>
</tr>
<tr>
<td>HLE 200 mg/kg BW</td>
<td>6.25±0.15±</td>
<td>6.25±0.15±</td>
</tr>
</tbody>
</table>

*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.

The immunomodulatory effect test on delayed-type hypersensitivity responses was carried out by providing antigen administration of HLE at doses of 50, 100, and 200 mg/kg BW was effective. The administration of HLE can increase total leukocytes compared to 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 leukocytes, 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.

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 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 CD3 and CD8 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.
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

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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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