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Immune-Enhancing Effect of Ethanol Extract of Rambutan (*Nephelium lappaceum* L.) Fruit Peel on phagocytosis index, Paw Edema Formation, and Antibody Titer in Mice

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ABSTRACT

Rambutan (*Nephelium lappaceum* L.) fruit peel contains flavonoids, phenolic compounds, and other phytoconstituents with health benefits and immunomodulatory effects. This study investigated the immunomodulatory effect of ethanol extract of rambutan peel (EERP) in mice using the carbon clearance method to evaluate innate/non-specific immune responses and delayed-type hypersensitivity (DTH). The antibody titer test was used to assess adaptive/specific immune responses. The experimental animals (mice) were divided into six groups. Group I (negative control) received oral doses of CMC-Na, while II and III received stimuno and prednisolone as positive control drugs. Groups IV-VI (test groups) were treated with 50, 100, and 200 mg/kg of EERP, respectively. The phagocytosis index value was obtained by the carbon clearance method using carbon suspension as an antigen. The percentage of paw edema was obtained by DTH test using SRBC (sheep red blood cells) as antigen. The secondary antibody titer was obtained using an antibody titer test using SRBC as an antigen by hemagglutination methods. The results showed that EERP at a dose of 100 mg/kg showed a greater immunostimulant effect than 50 and 200 mg/kg based on phagocytic index value 1.63±0.08, and increasing percentage of paw edema 53.77±14.87 % equivalent with positive control Stimuno. EERP dose of 200 mg/kg could boost the humoral immune response by increasing secondary antibody titers (1:2048). Phytochemical screening revealed that EERP contained saponin, flavonoid, and phenolic compounds. These findings showed that the EERP is a potential immunostimulant by increasing the phagocytic index, cellular inflammation and humoral immune responses.

Keywords: *Nephelium lappaceum* L., phagocytic index, antibody titer, Delayed Type Hypersensitivity

Introduction

The number of microorganisms in our environment and lifestyle-related stress can trigger immune system disorders. Currently, diseases of the immune system and autoimmune disorders are increasing. As a result, there is a need for immunomodulators (immunostimulants or immunosuppressants), natural or synthetic substances that can help modulate/regulate the immune system's balance. The two types of immune responses could be non-specific (innate) or specific (adaptive) immune responses.[1]

Nowadays, people turn to herbal medicine because they believe that plant remedies are relatively safe than synthetic medicine.[2] Reports have shown that food consumption may modulate and activate immune function and response. Consumption of rambutan fruit in the community left waste in the form of rambutan peel. The rambutan peel has been reported to contain flavonoids, tannins,[3] and phenolic compounds such as geraniin, ellagic acid, and corilagin.[4] Despite being a waste, rambutan peel has health benefits. Several kinds of research have shown that rambutan peel has *in vitro* antioxidant activity,[5] anti-hypercholesterolaemia, and antidiabetic activity in rats.[6]

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Chemical compounds such as flavonoids and phenolics in rambutan peel are suspected to have immunomodulatory effects.\Rambutan peel has the potential to be developed as a health supplement.[7]

There are several methods to test the immunomodulatory effect of plant extracts. Non-specific immunomodulatory activity can be measured by the carbon clearance method. It determines the rate carbon particles are eliminated from test animals' blood. In contrast, the specific immune response can be measured by delayed-type hypersensitivity and antibody titer assays. The greater the percentage difference in the thickness of the mice's legs, the greater the immunostimulant effect, which can activate the proliferation of T lymphocytes through cellular immune responses. The greater the antibody titer, the greater the immunostimulant effect. Both methods are relatively inexpensive and simple.[8] The DTH test is a cellular immune response involving T cells directly attacking antigens. The accumulation of leukocytes, macrophages, and other immune cells causes edema in the feet of mice as an inflammatory response.[9] This is due to the activation of Th1 (T helper) cells from lymphocyte cell proliferation. The antibody titer is a humoral immune response in which B cells proliferate into plasma cells to produce immunoglobulins or antibodies.[10] Previous research showed that rambutan peels at doses of 100 and 200 mg/kg had immunomodulatory effects in test animals induced by cyclophosphamide.[11]

The novelty of this research is using different extraction methods, namely maceration, different varieties of rambutan peel, and testing of its immunomodulatory activity without being induced by immunosuppressant agents.

Based on this, it is necessary to investigate EERP's immunomodulatory activity using non-specific parameters such as the carbon clearance test and specific parameters such as the DTH and

antibody titer test. Phytochemical screening was performed to ensure the chemical composition of EERP.

Materials and Methods

Preparation of EERP

The plant sample (*Nephelium lappaceum* L.) was collected from Surakarta, Central Java, Indonesia, on October 10th, 2020. The rambutan fruit sample was identified and authenticated at the Setia Budi University Surakarta Laboratory with voucher number 112/DET/UPT-LAB/23.12.2020. The peel of the rambutan fruit was dried and then macerated using 70% ethanol with simplicia in a ratio of 1:10 for three days. The filtrate was evaporated using a rotary evaporator at 60°C and then over a water bath to obtain a dry thick extract.

Phytochemical screening

Alkaloid test

The extract (0.5 g) was added to chloroform (10 mL) and a few drops of ammonia. The chloroform fraction was separated, and H_2SO_4 was added and divided into 3 parts. A white precipitate forms with Mayer's reagent, a brown colour precipitate with Wagner reagent, and a red precipitate with Dragendorff reagent, indicating the presence of alkaloids.[12]

Flavonoid Test

About 0.5g of the extract was mixed with 5 mL of distilled water and then filtered. Into the filtrate was added Mg powder, 1 mL of concentrated chloride acid, and 1 mL of amyl alcohol. The mixture was shaken vigorously and allowed to separate. The presence of flavonoid compounds was shown by the formation of yellow, orange, or red on the layer of amyl alcohol.[13]

Saponin test

About 0.5g of the extract was added to water, boiled for several minutes, and filtered. The filtrate was shaken vigorously. A stable foam that stays for about 10 min indicates the presence of saponin compounds.[13]

Tannin test

About 0.5g of the extract was added to 5 mL of distilled water and filtered. Three drops of 1% $FeCl_3$ was added to the filtrate. The formation of a blue or greenish-black colour indicated the tannins.[12]

Steroid and triterpenoid test

The extract (0.5g) was added to 5 mL of ethanol, then filtered and evaporated to dryness. About 1 mL of diethyl ether, 1 mL of concentrated H_2SO_4, and 1 mL of anhydrous CH_3COOH were added. A purple or red colour formation indicated the triterpenoids, while blue or green colour indicated the steroids.[12]

Test of immunomodulatory activity

Animals

Healthy Swiss male mice 8-10 weeks old, weighing 20-25 g, were obtained from the Faculty of Pharmacy, Universitas Muhammadiyah Surakarta (UMS). The animals were maintained under controlled light (12 h dark and 12 h light), under constant temperature (23±2°C), constant relative humidity (55±5 %), drinking aquadest *ad libitum*, and fed with standard rodent chow. Before the research, the test animals were acclimatized for seven days. The use of test animals and test protocols regarding the immunomodulatory activity of the EERP was submitted to the ethical clearance committee of the Faculty of Medicine at Universitas Muhammadiyah Surakarta and an ethical suitability letter with the number: 3055/A.1/KEPK-FKUMS/XI/2020 was issued.

Carbon clearance examination

Twenty-four mice were divided into 6 groups. EERP extract was administered at doses of 50, 100, and 200 mg/kg, 0.5% CMC Na (as negative control), stimuno (positive control immunostimulant), and prednisone (positive control immunosuppressant). All treatments were administered once a day orally for seven days. 25 µL blood sample was taken on day 8 through the tail vein (as a baseline). Then carbon suspension was injected intravenously through the tail. Carbon suspension was prepared by mixing 1.6 mL of carbon was suspended in 8.4 mL of 1% gelatin in NaCl solution.[14] Blood was taken at 5, 10, 15, 20, and 30 min after carbon induction.[15] 25 µL blood was added to 4 mL of 0.1% acetic acid. The carbon transmittance was measured with a spectrophotometer at 675 nm. The slope of each concentration vs time plotted between ordinate (100 - %transmittance) to abscissa (time) yields the coefficient of regression (Kr). The rate of carbon clearance was calculated as the phagocytic index (k).[16] Phagocytic index (k) =(Kr treatment group)/(Kr CMC Na group). Blood samples were collected from the animals and then sacrificed. The spleen and liver were taken and weighed. The relative organ weights were calculated by comparing them to the negative control group = [(organ weight (g)/(mice body weight (g)] x100%.

Making SRBC (Sheep Red Blood Cell)

An anticoagulant (Alsever's solution) was added to the Sheep's blood. Alsever's solution was prepared by dissolving 0.8 g sodium citrate, 0.05 g citric acid, 2 g dextrose, and 0.42 g NaCl in 100 mL of distilled water,[17] then sterilized in an autoclave at 121°C for 30 min. The blood sample was centrifuged at 3000 rpm for 10 min. The precipitate collected was washed three times using 0.9% NaCl solution. The washing process was repeated until the top layer was clear. The NaCl solution was separated to obtain 100% SRBC. This was diluted serially with 0.9% NaCl to obtain 50% and 10% SRBC suspension, respectively.[18]

Delayed-type hypersensitivity (DTH) test

The mice were divided into 6 groups (4 in each). On day 0, all groups were injected intraperitoneally with 10% SRBC; 0.1 mL/10 g, then from the first to the fifth day, mice were treated with CMC Na, Stimuno (6.5 mg/kg), Prednisone (25 mg/kg), and EERP at doses of 50, 100, and 200 mg/kg respectively. On the 6th day, the thickness of the right mice's feet was measured using a plethysmometer (T0). Furthermore, the mice were re-induced with 0.05 mL 10% SRBC subplantar. The mice's leg thickness was measured again on days 7 (T24) and 8 (T48). The difference in thickness of the right hind paw of mice before and after SRBC injection was referred to as a delayed-type hypersensitivity response.[19] The typical method of assessing DTH is an increase in foot and paw thickness.[20]

Antibody titer test

Six groups were used in this study (each group consisted of 4 mice). From the first day until the 12th day, mice were treated with CMC Na, Stimuno (6.5 mg/kg), Prednisone (25 mg/kg), and EERP at 50, 100, and 200 mg/kg, respectively. All mice were induced with 10% SRBC, 0.1 mL/10 g intraperitoneally twice on days 5 and 12. On the 13th day, blood was taken through the orbital vein and then centrifuged to separate the serum. The hemagglutination method was carried out using a double dilution, namely 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; 1:512; 1:1024; 1:2048; 1:4096 by preparing a 96 well round bottom microplate. 50 µL of 0.9% NaCl and 50 µL of serum were dropped into each well with 50 µL of 1% SRBC. The wells were left to stand at room temperature for 1 hour. The agglutination was then observed, and the antibody titer was determined. The antibody titer was calculated using the last dilution at which the antibody was still detectable by hemagglutination (visually visible). The value of hemagglutination was measured and expressed as a secondary antibody titer.[19]

Statistical analysis

The normality of the data on the volume of the thickness of the feet of mice was tested using the Shapiro-Wilk test, and the data homogeneity was tested using the Levene test. Because the data were not normally distributed and homogeneous, non-parametric tests, the Kruskal Wallis and the Mann-Whitney t-test were used with a 95% confidence level.

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Results and Discussion

The extraction yield was 9.66%, and the organoleptic test results of the EERP showed a viscous brown extract with a characteristic odour. The results of the phytochemical screening test (Table 1) showed that EERP did not contain alkaloids as reported in previous studies[11] This may be due to geographical location and differences in rambutan varieties. Various parameters, such as lymphocyte proliferation and phagocytosis of macrophages, can determine the immunomodulatory effect of a medicinal plant.[21] In this study, macrophage phagocytosis (the carbon clearance method), cellular immune responses were observed using the DTH method, and humoral immune responses were observed using the antibody titer method.

Non-specific immune response was determined by the carbon clearance test. Carbon acts as an antigen where carbon does not normally exist in the body. Carbon has a small molecular weight and stable particle sizes, so it does not clog blood vessels or lungs[22]. Because of its small molecular weight, carbon needs to be suspended with gelatin to increase its large molecular weight and then can function as an antigen. As a foreign object, carbon will be phagocytized by leukocyte cells, particularly macrophages, and neutrophils, found in the test animal's body. When carbon ink is injected intravenously, macrophages will phagocytose the carbon. Blood was taken from the tail vein after carbon ink injection at 5, 10, 15, 20, and 30 min, and the rate of carbon elimination was determined at 0 to 30 min (Figure 1). The linear regression line slope ratio between (100-% transmittance) versus time in the test and control groups was used to determine its phagocytic activity. The value of the linear regression line (k) slope indicates the calculated magnitude of the phagocytosis index. The immunomodulatory activities were classified according to their phagocytic index (Table 2).

Based on Table 2, the EERP showed efficacy as a strong immunostimulant at 100 mg/kg and moderate immunostimulation at 50 and 200 mg/kg. The test results of the positive control treatment with stimuno showed a phagocytic effect (k>1.5). The result validates the claim that stimuno has immunostimulant properties. In comparison, the positive control immunosuppressant (prednisone) had a phagocytic index value almost the same as the negative control.

Measurement of the relative weight of lymphoid organs (spleen) and liver can be seen in Table 3. As shown in Table 3, the group given an EERP dose of 100 mg/kg showed an increase in relative organ weights (spleen and liver). Measurements of the relative weight of lymphoid organs (spleen and liver) were carried out because lymphoid organs are needed for the maturation, differentiation, and proliferation of lymphocytes related to the immune system response.[23] The spleen is a secondary lymphoid organ. EERP increased spleen weight and organ index, but not significantly compared to the negative control. According to Wahyuningsih[24], an increase in the spleen organ index indicates a response to the proliferation of lymphocytes (B and T cells), which play a role in the immune system. B lymphocyte proliferation contributed to the increase in spleen weight (73.3%). The liver organ is not classified as a primary or secondary lymphoid organ. Still, the liver plays an important role in the immune response because it contains fixed macrophages, namely Kupffer cells.[14]

Testing of immunomodulatory activity on specific immune responses can use several methods, including DTH involving cellular immune responses. SRBC was used as the antigen and was injected into the mice in two stages. The first stage's goal is to activate Th1 cells via APC (Antigen Presenting Cells) and MHC (Major Histocompatibility Complex) class II. The second stage is re-exposure to activate macrophages and other inflammatory mediators by cytokines [14]. T cells are lymphocytes that play an important role in this cellular immune response. In this test, leukocytes, macrophages, and other immune cells accumulate as an inflammatory response after being sensitized to antigens (SRBC), causing inflammation in the feet of mice within 0-48 hours post-induction.[9] Table 4 shows the results of the EERP test on the DTH response.

The activity on specific immune responses can be seen using several methods, one of which is delayed-type hypersensitivity involving cellular immune responses. The antigen (SRBC) was injected into the mice in two stages, as described above. T cells are lymphocytes that play an important role in this cellular immune response. The DTH response test results for the 48th hour revealed differences in cellular-specific immune responses. In this study, the group of test animals given prednisone had the smallest difference in the edema volume (according to the theory), where prednisone is a corticosteroid drug used as an immunosuppressant because it has anti-inflammatory action. The immunosuppressive effect is produced by suppressing delayed-type hypersensitivity, namely by inhibiting the phagocytosis mechanism by macrophages and reducing T lymphocyte activity[25] so that it does not cause inflammation in the feet of mice.

The DTH response test results after 48 hours of treatment revealed differences in cellular-specific immune responses. The EERP 100 mg/kg produced the greatest change compared to the negative and positive controls (Table 4) with $p<0.05$. Based on these findings, it is possible to conclude that the EERP 100 mg/kg acts as an immunostimulant, activating T lymphocyte proliferation via cellular immune responses. The EERP at 50 and 200 mg/kg doses did not differ significantly from the negative control (it does not possess an immunostimulant effect). This is probably because the EERP 50 mg/kg was less effective due to the less stimulating bioactive content in lymphocyte cell proliferation activity that can provide cellular immune responses. There was a decrease in percentage change in the leg volume of mice at a dose of 200 mg/kg compared to EERP 100 mg/kg. The extract potential to stimulate T lymphocyte proliferation may have probably waned at this dose.

Besides, the antibody titer method can be used to observe the activity of the humoral immune response. B lymphocytes are involved in the humoral immune response that binds to antigens causing the proliferation of B cells leading to the production of antibodies by plasma cells. The role of antibodies as effectors is to eliminate antigens that macrophages can phagocytose.[26] The antibody titer formed was calculated by observing a hemagglutination reaction by looking at the highest dilution results of serum in mice.

Table 1: Phytochemical screening result of ethanol extract of rambutan peel

Group of the chemical compound	Results
Alkaloids	-
Tannins	+
Saponins	+
Triterpenoids and Steroids	-
Flavonoids	+

+ (positive): contains a group of chemical compounds; - (negative): did not contain a group of chemical compounds

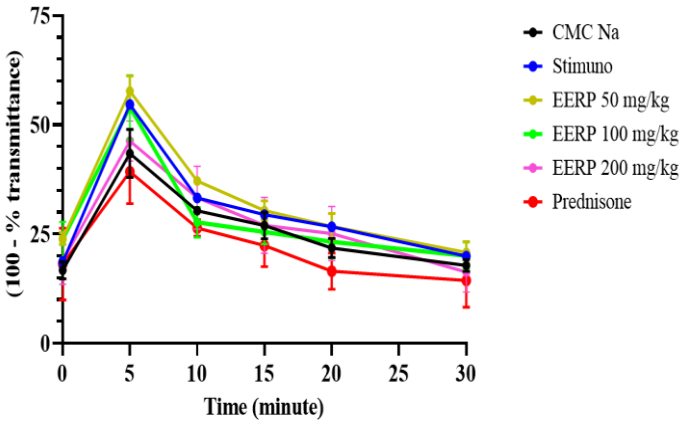

Figure 1: The rate of carbon elimination at 0 to 30 min after treatment of ethanol extract of rambutan peel (EERP) (n=4 mice in each group)

Table 2: Phagocytosis index value after administration of EERP for 7 days

Groups	Doses (mg/kg)	Regression coefficient (slope) (Kr)	Phagocytic index (k) $k = \frac{Kr\ treatment\ group}{Kr\ CMC\ Na\ group}$	Category of immunostimulating effect	% Phagocytic index change from negative control
EERP	50	-1.33 ± 0.07	1.37 ± 0.07	moderate	41.23
	100	-1.58 ± 0.07	1.63 ± 0.08[a]	strong	68.35
	200	-1.29 ± 0.28	1.33 ± 0.29	moderate	37.97
Stimuno	6.5	-1.51 ± 0.42	1.56 ± 0.43[a]	strong	61.03
Prednisone	5	-1.02 ± 0.14	1.05 ± 0.14	no effect	6.96
CMC Na	0	-0.97 ± 0.15	1.00 ± 0.16	no effect	3.29

Note: EERP = ethanol extract of rambutan peel. Each treatment group consisted of 4 mice. Percent change in phagocytic index [Kr treatment group - Kr CMC Na group) / Kr CMC Na group] x100%. [a] indicated significant difference with p<0.05 compared to the negative control. Phagocytic index (k) <1.2 (no immunostimulating effect), k=1.3-1.5 indicates a moderate immunostimulant, k >1.5 indicates a strong immunostimulant (Wagner, 1999)

Table 3: Relative organ weights (liver and spleen) after 7 days of treatment of EERP

Groups	Doses (mg/kg)	Weight of mice (g)	Liver organ (Mean ± SD)		Spleen organ (Mean ± SD)	
			Liver weight (g)	Relative organ weight	Spleen weight (g)	Relative organ weight
EERP	50	26.58 ± 2.57	1.09 ± 0.18	4.10 ± 0.70	0.11 ± 0.02	0.41 ± 0.11
EERP	100	27.05 ± 4.33	1.38 ± 0.13	5.10 ± 0.30	0.13 ± 0.04	0.48 ± 0.10
EERP	200	27.72 ± 0.40	1.22 ± 0.08	4.40 ± 0.20	0.12 ± 0.02	0.43 ± 0.10
CMC Na	0	29.74 ± 2.67	1.16 ± 0.08	3.90 ± 0.30	0.10 ± 0.01	0.34 ± 0.04
Stimuno	6.5	28.67 ± 4.75	1.27 ± 0.19	4.43 ± 0.40	0.15 ± 0.03	0.52 ± 0.10
Prednisone	5	26.43 ± 3.00	1.20 ± 0.21	4.54 ± 0.70	0.08 ± 0.04	0.31 ± 0.20

Note: EERP (Ethanol Extract of Rambutan Fruit Peel), this experiment used 4 replicates in each sample

When an antigen is first exposed to the body of a mouse, the resulting antibody is IgM, which represents the primary antibody titer. Antibody titer against mouse serum is tested by adding SRBC 1% to a 96-well plate to induce antigen-antibody interaction, thus forming a secondary reaction in the form of agglutination that can be observed visually.[27] The weakness of this study was not observing the primary antibody titer. Table 5 shows the results of the secondary antibody titer test. EERP doses of 100 and 200 mg/kg resulted in higher secondary antibody titer dilution than the negative control. In comparison, 50 mg/kg of EERP resulted in lower secondary antibody titer dilution than the negative control.

These findings suggest that the higher the dose, the more antigen binds to the antibody. The highest dilution occurred at the EERP dose of 200 mg/kg, and it has a greater ability to provide a humoral response than the positive control of Stimuno®. This result demonstrates that EERP at a dose of 200 mg/kg can stimulate the production of more antibodies. On the other hand, prednisone (immunosuppressants) had the smallest dilution compared to the other groups. It also occurs in the cellular immune response that prednisone decreases the immune response when used in normal mice. The carbon clearance and DTH test results showed a non-linear dose-response relationship where EERP at a 100 mg/kg dose increased the phagocytic index and DTH. In contrast, a higher dose (200 mg/kg) decreased phagocytosis activity (although still showing a moderate immunostimulating effect). This result may be because rambutan fruit peel can increase immune response only to a certain extent, and there may be a decrease at larger doses.

According to the findings of this study, EERP was proven to increase non-specific and specific immune responses in the form of cellular and humoral responses. The ability of EERP as an immunomodulator is thought to be caused by flavonoid and phenolic compounds. Polyphenol compounds can boost the immune response by increasing T lymphocyte proliferation and activation.[28] Several studies have found that some flavonoids and polyphenols improve immune response by increasing antibody production.[29] The type of polyphenol found in rambutan fruit peel is ethyl gallate.[30]

Conclusion

The ethanol extract of rambutan peel (EERP) contains flavonoids, tannins, and saponins. EERP doses of 100 and 200 mg/kg had a potential effect as an immunostimulant by enhancing immune and innate immune responses with an increase in the phagocytic index, cellular immune responses and humoral immune responses in normal mice that were immunized SRBC as an immune-challenge.

Conflict of Interest

The authors declare no conflict of interest.

Table 4: Average changes in the thickness (volume) of mice's feet in the delayed-type hypersensitivity test

Groups	% mice paw volume *Mean* ± SD (T48)
EERP 50 mg/kg	23.61 ± 13.25
EERP 100 mg/kg	53.77 ± 14.87[a]
EERP 200 mg/kg	34.79 ± 19.41
CMC-Na 0.5% (negative control)	20.63 ± 10.99
Prednisone (positive control immunosuppressant)	-28.75 ± 8.53[a]
Stimuno (positive control immunostimulant)	41.11 ± 8.46[a]

Note: The number of replicates was 3-4 mice per group, [a] indicated significant difference compared to the negative control with p<0.05, EERP: Ethanol Extract of Rambutan Fruit Peel. Negative (-): there is no change in the volume of the feet of mice T-48: 48th time after injection of SRBC (Sheep Red Blood Cell) antigen

Table 5: Antibody titer results after administration of ethanol extract of rambutan peel (EERP) mice on humoral immune response

Groups	Secondary antibody titer
EERP 50 mg/kg	1:128
EERP 100 mg/kg	1:1024
EERP 200 mg/kg	1:2048
CMC-Na 0.5%	1:256
Prednisone	1:2
Stimuno	1:512

Note: The number of test animals used is 4 mice in each group

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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