



## Immunomodulatory Effects of Ethanol Extract of Lime Peel on Phagocytosis Activity and Delayed-Type Hypersensitivity Response

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### Abstract

The natural human environment is inhabited by various organisms, including those harmful to the body such as bacteria, viruses, fungi, and other pathogens. This condition weakens the immune system's response to pathogens, making the body more susceptible to diseases such as rheumatoid arthritis, cancer, and other inflammations. In some cases, this condition can be fatal, leading to death. This study aims to examine the immunomodulatory effects of Ethanol Extract of Lime Peel on phagocytosis activity and delayed-type hypersensitivity response in experimental animals. The research methods include sample collection and processing, plant identification, extract preparation, and immunomodulatory effect testing. Phytochemical screening results showed the presence of alkaloids, glycosides, flavonoids, tannins, and steroids in the extract of the lime peel. Simplicia and ethanol extract of lime peel characterization yielded results meet the standards of the Indonesian Herbal Pharmacopoeia 2nd edition. Immunomodulatory effect testing was conducted through phagocytosis activity and delayed-type hypersensitivity response assays. The results showed that the extract at doses of 25, 50, and 100 mg/kgBW had immunostimulatory effects in enhancing phagocytosis activity, while doses of 17.5 mg/kgBW, 35 mg/kgBW, and 70 mg/kgBW significantly increased delayed-type hypersensitivity response compared to the negative control. These findings indicate the potential of ethanol extract of *Citrus aurantifolia* as an immunomodulator that can enhance the body's immune system. This study provides an important contribution to the development of immunomodulatory therapy derived from natural ingredients such as lime peel for managing immunity-related diseases with lower side effects.

### Introduction

The natural human environment is inhabited by various organisms, including those harmful to the body such as bacteria, viruses, fungi, and other pathogens. These pathogens are the main cause of diseases ranging from mild to fatal. However, the human body has a natural defense system against diseases called the immune system. The immune system is a complex network primarily consisting of white blood cells (lymphocytes, monocytes, neutrophils, and macrophages) and specific immune components (antibodies, proteins, and cytokines) that function to combat pathogens. The immune system response to pathogens is divided into innate and adaptive immune responses (Putri Prakarsa et al., 2024).

The body's defense system can weaken under certain conditions such as poor health, stress, genetic factors, nutritional deficiencies, previous infection history, smoking, alcohol consumption, age, HIV/AIDS, and others. These conditions weaken the immune system's response to pathogens, making the body more susceptible to diseases such as rheumatoid arthritis, cancer, and other inflammations. In some cases, this condition can be fatal, leading to death (Masniah et al., 2021). Therefore, immunomodulators are needed to enhance the immune system. Immunomodulators are substances that have the ability to modulate (enhance or decrease) both innate and adaptive immune responses to immunity-related diseases (Calder, 2021).

Immunomodulators are substances that have the ability to modulate (enhance or decrease) both innate and adaptive immune responses to immunity-related diseases. Immunomodulators are also used to restore immune response imbalance. Previously, many synthetic immunomodulatory agents have been developed with specific mechanisms but clinically failed to provide beneficial therapeutic actions due to bioavailability issues, stability problems, and serious side effects. Therefore, a new approach is needed for the development of new immunomodulators to manage immunity-related diseases with lower or no side effects (Behl et al., 2021).

Lime (*Citrus aurantifolia*) itself is a widely cultivated plant both globally and in Indonesia. The parts of the lime utilized are about 48% of the fruit as juice and flavoring in foods. The remainder (52%) includes the peel, seeds, and waste from lime usage in households or industries. Lime waste is recorded at 10,426,000 metric tons per year. Thus, strategies are needed for the utilization of this waste by creating value-added products, one of which is as medicinal ingredients (Phucharoenrak et al., 2023).

The peel of lime fruit has the potential to serve as an immunomodulator due to its flavonoid content, particularly hesperidin, which can act as an immunomodulator. Hesperidin has been shown to enhance the human body's immune system. Flavonoids found in citrus fruits exhibit anti-inflammatory effects and induce activation of immune cells, including T cells and B cells. The increase in the number of lymphocyte T cells, reduction in the proportion of B cells, and enhanced synthesis of IFN- $\gamma$  suggest that compounds like flavonoids possess immunostimulatory properties (Erjon, 2022).

Furthermore, Valdivieso-Ugarte et al. (2019) reported that essential oil in citrus peel significantly enhances immune response through several mechanisms such as improving B cell viability, inducing apoptosis, inhibiting pro-inflammatory cytokine secretion, and enhancing IL-10 secretion (Valdivieso-Ugarte dkk., 2019). Based on these findings, researchers are interested in applying secondary metabolites in lime peel to confirm their immunomodulatory effects in the processes of phagocytosis and delayed hypersensitivity response (Azwari et al., 2021).

Given the background above, the present study aims to investigate the immunomodulatory effects on phagocytosis activity in male mice and delayed hypersensitivity response to lime peel extract in male rats using the aforementioned dosage variations.

## Methods

This study was conducted experimentally to investigate the immunomodulatory effects of ethanol extract of lime peel on phagocytosis activity and delayed hypersensitivity in experimental animals. The research method included sample collection and processing, plant identification, extraction preparation, and immunomodulatory effect testing.

This experimental study encompassed the collection and processing of lime peel, simplicia characterization, phytochemical screening, preparation of ethanol extract of lime peel, experimental animal preparation, and immunomodulatory effect testing. Instruments used

included surgical tools, laboratory glassware, blender, oven, and UV-VIS spectrophotometry. The main materials used were lime peel and various chemicals such as ethanol, sulfuric acid, and *Staphylococcus aureus* bacterial solution. Plant collection was purposively conducted in the Laubaleng District, Karo Regency, North Sumatra. Lime peel samples were then processed into simplicia through drying, grinding, and storage (Luhurningtyas et al., 2020).

Macroscopic and microscopic examinations were conducted to observe the morphology and structure of lime peel simplicia. Additionally, determination of moisture content, water-soluble extract, ethanol-soluble extract, total ash, and acid-insoluble ash was carried out. Simplicia powder was extracted using the maceration method with 96% ethanol solvent. The extract was then evaporated to obtain a concentrated extract.

Phytochemical screening was performed to detect compounds such as alkaloids, flavonoids, glycosides, saponins, tannins, and steroids/triterpenoids in lime peel extract. Immunomodulatory effect testing was conducted through two approaches: phagocytosis activity testing and delayed hypersensitivity response testing in experimental animals. Careful preparation of experimental animals, controls, test materials, antigens, and suspension preparation for carbon clearance test and delayed hypersensitivity response test was carried out (Fristiohady et al., 2019).

This research method involved a series of procedures from sample collection to immunomodulatory effect testing of the extract. The experimental approach used is expected to provide an in-depth understanding of the immunomodulatory potential of lime peel extract. Research data were analyzed using SPSS version 22. The homogeneity and normality of research data were determined to decide the appropriate statistical analysis method. If the data were normal and homogeneous, ANOVA test was used to determine the mean differences among treatments. If differences were found, Post Hoc Tukey test was conducted to identify which variables had differences. However, if the data were not normal, Kruskal-Wallis test was used. The analysis was conducted with a significance value of  $p > 0.05$  (Malik et al., 2022).

## Result and Discussion

Plant identification conducted at the Herbarium Medanense (MEDA), Universitas Sumatera Utara, indicated that the plant used was lime (*Citrus aurantifolia* (Christm.) Swingle). Simplicia extraction of lime peel was performed using the maceration method with analytical grade ethanol solvent and concentrated using a rotary evaporator. From 300 grams of simplicia, 46.8 grams of concentrated ethanol extract was obtained, with a yield percentage of 15.6%.

### Results of Phytochemical Screening of Simplicia and Ethanol Extract of Lime Peel

Phytochemical screening of simplicia and ethanol extract from lime peel (*Citrus aurantifolia*) revealed the presence of alkaloids, glycosides, flavonoids, tannins, and steroids, while no saponin compounds were detected. Novriyanti et al. (2022) in their study on the screening of ethanol extract of lime peel stated that the ethanol extract of lime peel contains flavonoid, alkaloid, steroid, and tannin compounds. Based on the results obtained, it indicates that the extraction process proceeded well and effectively, as all secondary metabolites in the simplicia were extracted. The results of phytochemical screening of lime peel can be seen in Table 1.

Table 1. Results of phytochemical screening of lime peel simplicia and extract

No.	Parameter	Results	
		Simplicia	Extract
1	Alkaloid	+	+
2	Glikosida	+	+
3	Saponin	-	-
4	Flavonoid	+	+

5	Tanin	+	+
6	Steroid	+	+

Notes: + = Contains compound group

- = Does not contain compound group

### Results of Macroscopic and Microscopic Examination of Lime Peel Simplicia

The macroscopic examination results of lime peel simplicia revealed a fresh lime scent, greenish-yellow skin with a width of 1-1.5 cm and a length of 3-4 cm. Microscopic examination showed the presence of vascular bundles with ladder-type thickening, essential oil droplets, calcium oxalate crystals in prism shape, epidermis, and fibers.

### Characterization of Lime Peel Simplicia and Ethanol Extract

The characterization results of lime peel simplicia and ethanol extract (*Citrus aurantifolia*) are as follows: water content of 6.60% and 5.97%; water-soluble extract content of 28.48%; ethanol-soluble extract content of 21.09%; total ash content of 5.52% and 5.25%; insoluble ash content in acid of 0.31% and 0.08%. The characterization results are presented in Table 2.

Table 2. Characterization Results of Lime Peel Simplicia and Standardization Extract

No.	Parameter	Results	
		Simplicia	Extract
1	Water Content	6,60%	5,97%
2	Water-Soluble Extract Content	28,48%	-
3	Ethanol-Soluble Extract Content	21,09%	-
4	Total Ash Content	5,52%	5,16%
5	Acid-Insoluble Ash Content	0,31%	0,08%

Table 2 presents the characterization results of lime peel simplicia and ethanol extract, where the obtained results meet the criteria of the Indonesian Herbal Pharmacopoeia II edition.

### Carbon clearance test

The carbon clearance test is a non-specific response to determine the phagocytic activity of macrophage cells against carbon as a foreign substance. Carbon levels in the blood decrease over time due to phagocytosis events by leukocyte cells, especially neutrophils, monocytes, and macrophages (Sari et al., 2021). The carbon clearance test is conducted by injecting carbon ink into the bloodstream to measure the phagocytic mechanism of phagocytic cells (Kotala & Kurnia, 2022).

### Carbon elimination rate

The carbon elimination rate is a method used to measure phagocytic activity in mice. Carbon is used as an exogenous antigen compound, and the body responds by phagocytosis. The reduction in the number of carbon particles in the plasma represents the carbon particle elimination rate from the test animals (Erjon, 2022). The carbon elimination rate is measured at the 5th, 10th, 15th, and 20th minutes. The results of carbon elimination rate in the blood are shown in Figure 1.

Based on the statistical test results, it is evident that at the 5th minute, several treatment groups showed significant differences ( $p > 0.05$ ). At the 5th minute, CMC Na did not significantly differ from all treatment groups. Imboost® exhibited significant differences compared to extract doses of 25 mg/kgBW and 50 mg/kgBW, but not significantly different from extract doses of 100 mg/kgBW. At the 10th minute, CMC Na significantly differed from all treatment groups ( $p < 0.05$ ). Imboost® showed significant differences compared to extract doses of 25 mg/kgBW

and extract doses of 50 mg/kgBW, but not significantly different from extract doses of 100 mg/kgBW. The extract doses for 25 mg/kgBW significantly differed from the CMC Na and Imboost® groups but not significantly different from extract doses of 50 mg/kgBW and extract doses of 100 mg/kgBW. The extract doses for 50 mg/kgBW significantly differed from the CMC Na and Imboost® groups but not significantly different from extract doses of 25 mg/kgBW and extract doses of 100 mg/kgBW. The extract doses for 100 mg/kgBW significantly differed from the CMC Na group but not significantly different from the Imboost®, extract doses of 25 mg/kgBW, and extract doses of 50 mg/kgBW groups (Valdivieso-Ugarte et al., 2019).

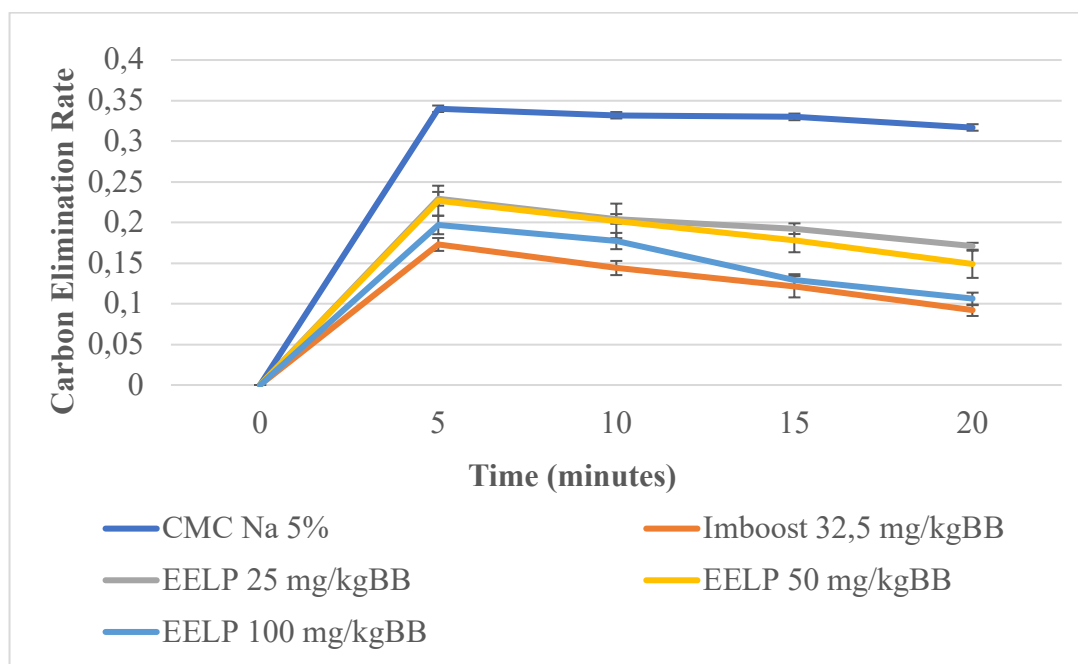


Figure 1. Carbon elimination rate results in mouse blood

Notes: EELP = Ethanol Extract of Lime Peel

Based on the statistical test results, the absorbance value of the carbon wavelength in the blood decreased over each time interval, indicating that each concentration of the test extract had an immunostimulatory effect. The use of various concentrations of the test extract in this study aimed to determine the relationship between increasing extract concentration and the activity of carbon reduction in the blood. In the conducted study, a decrease in absorbance values was observed in all test formulation groups compared to the negative control group. The greatest decrease in absorbance values was found at a dose of 100 mg/kgBW. This demonstrates an increase in phagocytosis activity in each group of test extract formulations (Dogan, 2022).

Table 3. Effect of ethanol extract of lime peel on carbon elimination constants (Mean  $\pm$  SEM)

No	Treatment Group	Carbon Elimination Constant
1	CMC Na 0,5%	0,0020 $\pm$ 0,0001 (+)
2	Extract doses of 25 mg/kg BW	0,0084 $\pm$ 0,0000 (*+)
3	Extract doses of 50 mg/kg BW	0,0125 $\pm$ 0,0021 (*)
4	Extract doses of 100 mg/kg BW	0,0180 $\pm$ 0,0006 (*)
5	Imboost 32,5 mg/kg BW	0,0183 $\pm$ 0,0022 (*)

Notes: \* = significantly different from the negative control (CMC Na 0.5% Group)  
+ = significantly different from the positive control (Imboost Group 32.5 mg/kgBW)

Absorbance data were used to calculate the phagocytosis constant values. The elimination constant is one of the parameters used to determine the rate of phagocytosis. The larger the elimination constant value, the higher the rate of carbon clearance, indicating a faster phagocytic cell process (Kirana et al., 2023).

### Phagocytosis Index

The phagocytosis activity test using the carbon clearance method provides an overview of the non-specific immune system in the process of phagocytosis against foreign particles in the blood. The carbon clearance method is used to measure the activity of phagocytic cells in killing pathogenic organisms that enter the body. Phagocytosis is widely used as an immunological parameter to evaluate the body's immune function. Assessment of the ability or activity of phagocytosis in eliminating carbon particles is expressed as the phagocytosis index. An increase in carbon clearance index indicates an improvement in the phagocytic function of mononuclear macrophages and non-specific immunity (Asfianti et al., 2022). The results of the phagocytosis index can be seen in Table 4.

Table 4. Effect of ethanol extract of lime peel on phagocytosis index results (Mean  $\pm$  SEM)

No	Treatment Group	Carbon Elimination Constant
1	CMC Na 0,5%	1,3100 $\pm$ 0,0877 (+)
2	Extract doses of 25 mg/kg BW	2,5849 $\pm$ 0,1912 (*+)
3	Extract doses of 50 mg/kg BW	3,0583 $\pm$ 0,2511 (*+)
4	Extract doses of 100 mg/kg BW	3,4510 $\pm$ 0,1652 (*+)
5	Imboost 32,5 mg/kg BW	4,4337 $\pm$ 0,2633 (*)

Notes: \* = significantly different from the negative control (CMC Na 0.5% Group)  
+ = significantly different from the positive control (Imboost Group 32.5 mg/kgBW)

Table 4 shows that the phagocytosis index of CMC Na 0.5%, extract doses of 25 mg/kgBW, extract doses of 50 mg/kgBW, extract doses of 100 mg/kgBW, and Imboost® 32.5 mg/kgBW are 1.3100, 2.5849, 3.0583, 3.4510, and 4.4337, respectively. After obtaining the phagocytosis index data, the data were processed using One-Way ANOVA statistical test with SPSS, followed by further analysis using Post Hoc Tukey. The phagocytosis index after the extract administration (Y. et al., 2020).

Based on the average phagocytosis index values, it indicates the phagocytosis activity of phagocytic cells against carbon ink as an antigen due to the influence of the extract suspension administration. Thus, it can be concluded that extract doses of 25, 50, 100 mg/kgBW can increase phagocytosis activity. Imboost® 32.5 mg/kgBW has the highest phagocytosis index compared to other treatments. The phagocytosis index of extract doses of 25, 50, 100 mg/kgBW show phagocytosis index values greater than one (IF>1), where if the average phagocytosis index value is greater than one (IF>1), it indicates that the test substance has immunostimulatory abilities. Increasing the dose of extract administration also shows an increase in phagocytosis index values (Khuluq et al., 2021).

### Stimulation Index

The stimulation index is the result of comparing the test group with the control group. A substance is considered immunostimulatory if the stimulation index is greater than 1 and immunosuppressive if the stimulation index is less than 1 (Denaro et al., 2021). The stimulation index after extract administration are shown in Table 5.

Table 5. Effect of ethanol extract of lime peel on stimulation index

No	Treatment Group	Stimulation Index
1	Imboost 32,5 mg/kgBW	3.3844

2	Extract doses of 25 mg/kg BW	1.9731
3	Extract doses of 50 mg/kg BW	2.3345
4	Extract doses of 100 mg/kg BW	2.6343

The stimulation index of Imboost® 32.5 mg/kgBW, extract doses of 25 mg/kgBW, extract doses of 50 mg/kgBW, and extract doses of 100 mg/kgBW are 3.3844, 1.9731, 2.3345, and 2.6343, respectively. The extract suspensions at doses of 25, 50, and 100 mg/kgBW show a dose-dependent relationship with the stimulation index values for all three doses, indicating that as the dose increases, the stimulation index value also increases. This suggests that extract at doses of 25, 50, and 100 mg/kgBW acts as an immunostimulant (Chen et al., 2020).

Similar findings were reported in the study by Pucharoenrak et al. (2023), which stated that lime peel contains flavonoids, tannins, steroids/terpenoids, alkaloids, and several phenolic compounds. These compounds have been proven to have activity in modulating the immune system. In the study by Kasim et al. (2020), it was explained that lime peel can modulate the immune pathways, especially the TLR-4 signaling pathway (Syachriyani et al., 2023). Furthermore, in the study by Valdivieso-Ugarte et al. (2019), it was reported that essential oil in lime peel significantly enhances immune response through several mechanisms such as increasing B cell viability, enhancing apoptosis, inhibiting pro-inflammatory cytokine secretion, and increasing IL-10 secretion (Borowicz et al., 2020).

### Delayed-type hypersensitivity test

Delayed-type hypersensitivity response is a rapid in vivo manifestation of T cell-dependent immune response to foreign antigens. The delayed-type hypersensitivity test is a evaluation of immunomodulatory effects related to specific immune response. Delayed-type hypersensitivity response is a cellular immune response involving activation of Th cells that release pro-inflammatory cytokines and enhance macrophage activity, characterized by swelling of the test animal's paw (Bondonno et al., 2020).

The stimulation index is the result of comparing the test group with the control group. A substance is considered immunostimulatory if the stimulation index is greater than 1 and immunosuppressive if the stimulation index is less than 1 (Denaro et al., 2021). The stimulation index after extract administration are shown in Table 5.

Table 6. Effect of ethanol extract of lime peel on rat paw swelling volume data

No	Treatment Group	Rat Paw Volume (ml) (Mean ± SEM)
1	CMC Na 0,5%	0.292 ± 0.1059 (+)
2	Extract doses 17,5 mg/kgBW	2,096 ± 0.6202 (*)
3	Extract doses 35 mg/kgBW	2,462 ± 0.4314 (*)
4	Extract doses 70 mg/kgBW	2,902 ± 0.4595 (+*)
5	Levamisole 25 mg/kgBW	1.688 ± 0.5447 (*)

Notes: \* = significantly different from the negative control (CMC Na 0.5% Group)

+ = significantly different from the positive control (Levamisole Group 25 mg/kgBW)

Based on Table 6, there is an increase in rat paw volume induced by *Staphylococcus aureus*. It was observed that the average increase in rat paw volume increased with increasing extract dose compared to the control group. The extract groups at doses of 17.5 mg/kgBW, 35 mg/kgBW, and 70 mg/kgBW had larger paw swelling volumes than those obtained in the Levamisole 25 mg/kgBW control group. This is suspected because ethanol extract of lime peel contains many secondary metabolites, one of which is flavonoids that have been extensively studied for their ability to enhance the immune system to stimulate inflammatory reactions and activate Th<sub>1</sub> cells, which may proliferate and release cytokines causing increased blood vessel

permeability, vasodilation, macrophage accumulation, and ultimately inflammation (Lu et al., 2022). Levamisole itself has no significant effect on T cell or natural killer (NK) cell activation and cannot enhance lymphocyte proliferation but can increase IL-12 and IL-10 production (Asfianti et al., 2022).

## Conclusion

Based on the testing of the immunomodulatory effects of ethanol extract of lime peel on phagocytosis activity and delayed-type hypersensitivity response, the conclusions drawn from this study include the following: Ethanol extract of lime peel at doses of 25, 50, and 100 mg/kgBW has immunostimulatory effects in enhancing phagocytosis activity in male white mice injected with different carbon suspensions significantly different compared to the negative control CMC Na 0.5%. Ethanol extract of lime peel at doses of 17.5 mg/kgBW, 35 mg/kgBW, and 70 mg/kgBW has immunostimulatory effects that increase delayed-type hypersensitivity response in male white rats significantly different compared to the negative control CMC Na 0.5% and positive control Levamisole 25 mg/kgBW because it is shown a greater effect than the positive control.

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