Immunostimulatory activity of avocado oil in mice 
(*Mus musculus*)

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Abstract

Avocado fruit contains bioactive compounds that have the potential to act as an immunomodulator. This study aims to identify the ability of avocado oil as an immunomodulator based on the macrophage phagocytic activity and index of mice injected with nonpathogenic *Staphylococcus aureus* and to determine the most effective dose as an immunomodulator. This study used 30 male Deutschland Denken Yoken (DDY) mice, which were divided into five groups: placebo as negative control (mineral water), positive control (commercial immunomodulator containing Echinacea purpurea extract), and avocado oil 1 g/kg BW, 3 g/kg BW, and 5 g/kg BW. The treatment was carried out orally once a day for 14 days. Mice were induced by nonpathogenic *Staphylococcus aureus* on day 15 intraperitoneally, and after one hour, mice were euthanized to collect the peritoneal fluid. Peritoneal fluid smear preparations were made before active macrophages and phagocytosed *Staphylococcus aureus* were observed under a microscope. Phagocytic activity and phagocytic index were calculated. Avocado oil 5 g/kg BW showed the highest phagocytic activity and phagocytic index results with values of 71.00% ± 5.40 and 2.79 ± 0.14, respectively. Avocado oil enhances non-specific immune responses through macrophage phagocytic activity and index, demonstrating its immunostimulatory potential.

Keywords: avocado oil | immunomodulator | macrophage | phagocytic activity | phagocytic index

Introduction

Infectious disease is caused by the entry and multiplication of microorganisms in the body that can infect other prone individuals. Microorganisms are a broad group of microscopic organisms consisting of one or many cells, such as bacteria, fungi, parasites, and viruses. Infectious disease is a significant public health problem in developed and developing countries. According to the World Health Organization (WHO), infectious diseases are the leading cause of death in children. According to WHO (2015), the mortality rate for children under five years in Indonesia is 1–20% caused by infectious diseases. The high mutation rate of microorganisms is also the reason for the rapid transmission of infectious diseases.

Microorganisms, especially bacteria and viruses that can harm humans, can be found in homes, the surrounding environment, and anywhere else. The body has a defense mechanism to dispel, ward off, or eliminate these bacteria and viruses from entering the body. This defense mechanism is called the body’s immune system, which forms the body’s ability to fight disease by rejecting various...
foreign objects that enter the body to avoid disease (Oktavia & Muksin, 2021). Some conditions that can weaken a person’s immune system are aging, malnutrition, disease, and consumption of certain drugs. Therefore, the function of the immune system needs to be maintained so that the immune system remains optimal (Setyoningsih et al., 2021).

Immunomodulators have been used to restore immune imbalances. Immunomodulators are natural or synthetic substances that can enhance or inhibit the functions of the innate and adaptive immune system (Jantan et al., 2019). Many synthetic immunomodulator drugs with various mechanisms have been discovered and developed, but these drugs still have side effects potential for human health. Herbal medicines and their active metabolites provide alternative potential as sustainable therapies for various immunological disorders by modulating the immune response (Mohamed et al., 2017); therefore, searching for herbal medicine with fewer side effects is necessary.

Avocado (Persea americana) is one of the many known fruits found in Indonesia and has the potential as an herbal. Avocado is a very nutritious fruit, rich in unsaturated fatty acids, and has various compounds with antioxidant, anticancer, antimicrobial, and anti-inflammatory properties (Báez-Magaña et al., 2019). Avocado oil is obtained from the extraction of avocado pulp and contains bioactive compounds, including vitamins A, B, C, E, phytosterols, and polyphenols (Woolf et al., 2009). The abundance of bioactive compounds contained in avocado oil has the potential to act as an immunomodulator agent. This potential prompted the authors to test the activity of avocado oil as an immunomodulator.

**Methods**

**Animals**
This research procedure obtained approval from the Animal Ethics Commission of the School of Veterinary Medicine and Biomedical Sciences, IPB University, with number 019/KEH/SKE/III/2023 on March 7, 2023. Thirty male DDY strain *Mus musculus* mice, 25–30 g of weight and approximately eight weeks old, were used in this research. The mice were ensured to be healthy during the acclimatization period. The mice were given ad libitum feed and water and placed in cages measuring 35×25×10 cm. Rice husks were provided in the cages, and the cages were cleaned twice a week. The mice were given ivermectin anthelmintic at a dose of 0.04 mg/kg BW diluted with mineral water in the mouse drinking bottles daily during the acclimatization period. The mice were acclimated for seven days before the start of the treatment.

**In vivo testing**
The mice were divided into five groups, with six in each group. The five treatment groups were as follows: Group A: mineral water at a volume of 5 g/kg BW (150 µL) as a negative control (placebo); Group B: commercial immunomodulator (*Echinacea purpurea*) at a 0.250 g/kg BW (150 µL) as a positive control; Group C: avocado oil at a dose of 1 g/kg BW (30 µL); Group D: avocado oil at a dose of 3 g/kg BW (90 µL); Group E: avocado oil at a dose of 5 g/kg BW (150 µL). The treatments were administered once a day for 14 days by orally administering the preparations to the mice using a micropipette. The oral administration was performed by handling the mouse at the back of the neck and looping the mouse’s tail around the pinky finger.

**Collection and preparation of peritoneal fluid samples**
On the 15th day, mice were induced with 1 mL of nonpathogenic *S. aureus* at a concentration of 10⁸ CFU/mL via intraperitoneal injection and left for one hour. After one hour, the mice were euthanized by cervical dislocation and dissected to collect the peritoneal fluid samples using a 1 mL syringe. Before collecting the peritoneal fluid, 1 mL of physiological saline (NaCl) was added to the abdominal cavity, and the mice were gently shaken to mix the physiological saline with the peritoneal fluid. The peritoneal
fluid samples were collected and transferred into microtubes, then centrifuged at 5,000 rpm for three minutes to separate the supernatant from the pellet (sediment).

Microscope slides were cleaned with 70% alcohol and wiped until clean, dry, and greased-free. The pellet from the centrifuged peritoneal fluid sample was collected using a micropipette and dropped onto one side of the microscope slide. A second slide was placed at approximately a 30-degree angle to the first slide, touching the droplet of peritoneal fluid, and then pushed along the surface of the first slide to create a thin and even layer. The prepared peritoneal fluid slides were left to dry.

**Staining and evaluation of peritoneal fluid slides**

The dried slides were fixed in a solution of methanol for five minutes. The slides were then immersed in a 10% Giemsa solution and left for 20 minutes. The slides were rinsed with water and dried. Once dried, the slides were labeled according to the treatment groups.

The observation was performed under a microscope at a magnification of 1000×. Immersion oil was added to the microscope slide to enhance the visibility of macrophage cells. The observation continued until 100 macrophages were counted in total on each slide. During observation, macrophages were differentiated between active and passive. Active macrophages were characterized by phagocytosed *S. aureus* bacteria within the active macrophages, as seen in Figure 1. *S. aureus* was chosen as the antigen because it is a gram-positive bacterium, allowing it to bind more clearly to Giemsa stain and having a round (cocci) shape, which facilitates the process of calculating phagocytic activity under the microscope (Haeria et al., 2021).

The results of the five treatment groups for the phagocytic activity and index of macrophages are listed in Table 1. It is showed that there are significant differences between several treatment groups in terms of macrophage phagocytic activity. All groups treated with avocado oil (at doses of 1 g/kg BW, 3 g/kg BW, and 5 g/kg BW) showed a significant increase in phagocytic activity compared to the negative control group. The groups given avocado oil at doses of 3 g/kg BW and 5 g/kg BW did not show a significant difference in phagocytic activity compared to the positive control group. The highest phagocytic activity was found in the group by 100 counted macrophages and multiplying by 100%. The phagocytic index was determined by dividing the total number of *S. aureus* bacteria phagocytosed by active macrophages by the number of active macrophages in 100 counted macrophages (Lin et al., 2013).

The measured observation data were the macrophage phagocytic activity and index. The distribution of data obtained for the macrophage phagocytic activity and index of was tested for normality. The data has a normal distribution and was analyzed using Analysis of Variance (ANOVA) with significant differences of p<0.05, followed by the Tukey test. Data analysis was performed using Minitab 21® software.

**Results**

The immunomodulatory properties of avocado oil were tested by measuring the phagocytic activity and index of macrophages in male mice. Active macrophages were characterized by one or more phagocytosed *S. aureus* bacteria within the active macrophages, as seen in Figure 1. *S. aureus* was chosen as the antigen because it is a gram-positive bacterium, allowing it to bind more clearly to Giemsa stain and having a round (cocci) shape, which facilitates the process of calculating phagocytic activity under the microscope (Haeria et al., 2021).

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treated with avocado oil at 5 g/kg BW dose with an average of 71.00 ± 5.40%. In contrast, the negative control group showed the lowest phagocytic activity with an average of 44.83 ± 3.71%.

Similarly, it is known that there are significant differences between several treatment groups in terms of macrophage phagocytic index. The groups treated with avocado oil at doses of 3 g/kg BW and 5 g/kg BW showed a significant increase in phagocytic index compared to the negative control and positive control groups. The highest phagocytic index was found in the group treated with avocado oil at 5 g/kg BW dose with an average of 2.79 ± 0.14. This result is significantly different from the other four groups, while the lowest phagocytic index was found in the negative control group with an average of 1.70 ± 0.05.

### Discussion

The increase in phagocytic activity and index (Table 1) is directly proportional to the dosage of avocado oil administered. This result means that the higher of the avocado oil dosage, the higher the phagocytic activity and index values. According to Rosnizar et al. (2021), an increase in macrophage phagocytic activity and index indicates improved immune response.

Macrophages are the main mononuclear phagocytic cells involved in phagocytosing microorganisms and other foreign molecular complexes in tissues. These cells develop from monocytes (circulating monocytes) produced in the bone marrow. Monocytes migrate to tissues and differentiate into macrophages, then reside in tissues as resident macrophages (Baratawidjaja, 2006).

### Table 1 The effect of avocado oil administration on phagocytosis activity and phagocytosis index (Mean±SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (-)</th>
<th>Control (+)</th>
<th>Avocado oil (g/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytosis activity (%)</td>
<td>44.83 ± 3.71</td>
<td>70.17 ± 4.96</td>
<td>61.67 ± 4.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phagocytosis index</td>
<td>1.70 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.82 ± 0.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.90 ± 0.24&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters on the same row indicate significant differences (p<0.05).

Figure 1 Peritoneal fluid sample. (A) Passive macrophage; (B) Active macrophage; (C) Phagocytosed *S. aureus* within the active macrophage; (D) *S. aureus* outside macrophages. Giemsa staining, magnification of 1000×.
Exposure to *S. aureus* bacteria triggers infection in animals because *S. aureus* contains lipoteichoic acid (LTA) that affects the immune response by activating the expression of cytokines such as IFN-γ and TNF-α (Fournier & Philpott, 2005). During the infection process, T lymphocytes produce a variety of chemokines that attract macrophages to the site of need and activate them (Wahyuni et al., 2019).

As the first line of defense against the entry of antigens into the body, the non-specific immune system, namely macrophages, will destroy antigens through phagocytosis. Macrophages phagocytose antigens by extending their plasma membrane and engulfing the antigen by surrounding it with the membrane. Once inside the macrophage cell, the antigen is stored in a phagosome and fuses with a lysosome. Lysosomes contain enzymes and acids that kill and digest particles or organisms (Lekstrom-Himes & Gallin, 2000). Active macrophages release several vital substances, including enzymes, lysozyme, elastase, collagenase, complement, and cytokines (Wahyuni et al., 2019). Some cytokines secreted by macrophages in response to pathogens include interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 12 (IL-12), Tumor Necrosis Factor-alpha (TNF-alpha), and interleukin 8 (IL-8) chemokine (Abbas & Lichtman, 2010).

Immunomodulators work by modifying the immune system response through the increase (immunostimulation) or decrease (immunosuppression) in the production of serum antibodies and immune cells, such as white blood cells, macrophages, neutrophils, natural killer (NK) cells, and cytotoxic T lymphocytes (Avorn, 2011). Based on this research, the administration of avocado oil has been shown to increase phagocytic activity and index of macrophages in mice. This result proves the immunomodulatory activity of avocado oil as an immunostimulant.

Avocado has a higher lipid content compared to other fruits. Oleic acid, a monounsaturated fatty acid, is the primary fatty acid in avocado oil. Several publications have shown the potential of oleic acid as an immunostimulant to induce immune responses. Oleic acid can modulate signaling pathways in inflammatory responses, cell differentiation, and immune cell proliferation (Hidalgo et al., 2021). Natnan et al. (2022) demonstrated that oleic acid supplementation could increase lysozyme activity and phagocytosis. Another study by Li et al. (2019) revealed that oleic acid showed increased expression of proinflammatory genes and increased activity of related immune proteins and enzymes, such as TNF-alpha, IL-1beta, and cyclooxygenase-2 (COX-2). Avocado oil contains various vitamins, including vitamin A, riboflavin, pyridoxine, pantothenic acid, folate, thiamine HCl, ascorbic acid, niacin, choline, biotin, and vitamin E (tocopherol) (Sari et al., 2021). Supplementation of vitamin E in animals has been shown to improve the function of T-cell mediation, including T-cell differentiation in the thymus, lymphocyte proliferation, IL-2 production, and helper T-cell activity (Ortega-Arellano et al., 2019). The innate immune functions, including NK cell activity and macrophage phagocytic capacity, also increase with vitamin E supplementation (Bhattacharyya et al., 2010).

Phenolic compounds (including phenolic acids and hydroxycinnamates, flavonoids, and condensed tannins), carotenoids, α, β, γ, and δ-tocopherol, acetogenin, monounsaturated fatty acids, and polyunsaturated fatty acids are proven to be the major antioxidant components found in avocado (Wang et al., 2010). Flavonoids act on lymphokines produced by T cells, stimulating phagocytic cells to perform phagocytosis responses (Aldi et al., 2014). Flavonoids enhance the activation of effector cells, such as lymphocytes and macrophages, which produce and release IL-1, IL-6, IL-12, and TNF-α. Higher doses of flavonoids make phagocytic cells more active in phagocytosing bacterial cells, destroying and degrading more bacteria (Zalizar, 2013).
β-sitosterol is the main phytosterol found in avocado oil, followed by campesterol and stigmasterol (Duester, 2001). β-sitosterol is essential in strengthening the immune system and suppressing human immunodeficiency virus and other infections. β-sitosterol has been shown to enhance lymphocyte proliferation and the activity of natural killer cells in destroying pathogens (Bouic et al., 1996). Research by Wahdaningsih et al. (2021) revealed that β-sitosterol isolates demonstrated immunostimulant activity with increased macrophage phagocytic activity and significant nitric oxide (NO) production. NO is one of the reactive nitrogen species (RNS) secreted by macrophages to facilitate phagocytosis of antigens, including bacteria, fungi, and viruses (Abbas et al., 2012).

Tannin compounds affect physiological activities by stimulating antitumor and antibacterial phagocytic cells (Shashirekha et al., 2015). Administration of tannins increases IL-12 production, which stimulates NK cells and helps differentiate Th cells into Th1. Th1 cells secrete IFN-γ, which activates macrophages to produce reactive oxygen species (ROS) (Arram et al., 2014). ROS are reactive molecules chemically derived from oxygen molecules and are fundamental for macrophages to phagocytose antigens (Canton et al., 2021).

Conclusion
Administration of avocado oil can enhance the phagocytic activity and index of macrophages in the peritoneal fluid of mice after induction with nonpathogenic S. aureus. The increased activity and phagocytic index of macrophages demonstrate that avocado oil has immunomodulatory capabilities as an immunostimulant. Avocado oil at a dose of 5 g/kg body weight showed the highest macrophage phagocytic activity and index.

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Author contributions: EAJ performed the experiment, analyzed the data, and wrote the paper; AAM and AA conceived and designed the experiments.

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