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Immunostimulatory Effects of *Zingiber ottensii* Rhizome Extract on Mouse Lymphocytes and Peritoneal Macrophages

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ABSTRACT

Zingiber ottensii (*bangle hantu*) is a member of the Zingiberaceae family, known for its pharmacological effects. Several studies have demonstrated immunomodulatory potential of Zingiberaceae rhizomes, including *Zingiber officinale*, *Zingiber zerumbet*, and *Zingiber cassumunar*. This study aimed to evaluate the immunomodulatory effects of *Z. ottensii* extract on mouse peritoneal macrophages and lymphocytes. The extract was prepared using a maceration method with 96% ethanol (1:6) and the extract compound was observed by phytochemical screening with thin layer chromatography (TLC). Phagocytotic macrophage activity was quantified using a phagocytotic assay, which use mouse peritoneal macrophages. Lymphocyte proliferation was assessed with the MTT assay and absorbance measured at 595 nm. TLC results, showed that *Z. ottensii* extract tested positive for flavonoids and terpenoids. The *Z. ottensii* extract stimulated macrophage phagocytosis activity, significantly increasing the phagocytotic index at 25 and 50 µg/mL concentrations compared to the control. Additionally, significant lymphocyte proliferation was observed with the treatment of the extract. The *Z. ottensii* extract may be developed for adjuvant therapy to enhance the immune responses, offering a promising natural approach to immunomodulation.
Keywords: immunomodulatory effect; lymphocyte; macrophage; *Zingiber ottensii*

INTRODUCTION

Infectious agents are frequently introduced to the human body, leading to illness. The immune system can prevent these infectious pathogens from getting infiltrating an causing disease. The immune system plays a crucial role in protecting body cells from infections and chronic illnesses. An immunomodulator that can affect both the humoral and cellular immune systems is required to enhance and control immunity. Immunomodulators were developed in response to the prevalence of immune system disorders, which affect both humoral and cellular immunity (Abbas et al., 2022).

Currently, research and development of immunomodulators are encouraged and can be utilized as complementary therapies (Parham, 2014). Numerous investigations on immunomodulators derived from natural sources, including an extract from *Echinacea purpurea*, have demonstrated the ability to enhance the production of Th1 and Th2 cytokines (Aida Rahmadani & Lestari, 2021; Owen et al., 2013). Furthermore, using *Phyllanthus niruri* water extract as an immunostimulant can boost neutrophil activation, antibody responses, cell proliferation, and macrophage phagocytosis (Putri et al., 2022).

One traditional medicine that can be developed as an immunomodulatory agent is the rhizome of *bangle hantu* (*Zingiber ottensii*) which has been proven to have anti-cancer activity on MCF-7 cells (Sinaga & Wiryanti, 2013). The rhizome of *Z. ottensii* contains the flavonoid compound kaemferol, which has anti-inflammatory and anti-cancer activity (Chien et al., 2008; Han et al., 2018; Imran et al., 2019). Given these properties, *Z. ottensii* rhizomes have the potential to be developed as phytotherapy. Several studies have demonstrated that Zingiberaceae rhizomes possesses immunomodulatory potential. For instance, *Zingiber zerumbet* has been shown to exhibit immunomodulatory activity (Hidayah & Indradi, 2020), and the zerumbon compound found in Zingiberaceae also possesses immunomodulatory properties (Keong Yeap et al., 2010).

This investigation focuses on the immunomodulatory effects of *Z. ottensii* rhizome on the cellular immune response, specifically phagocytotic macrophage activation and lymphocyte proliferation. The rhizome of *Z. ottensii* may have a positive impact on human health and

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the prevent illnesses related to the immune system. This study aims to provide a more comprehensive understanding of *Z. ottensii*'s pharmacodynamic action, generating scientific data that could support the findings of previous study.

MATERIALS AND METHODS

Materials

Bangle hantu (*Zingiber ottensii*) rhizomes were collected from Lembang, West Java, Indonesia, and authenticated by the Laboratory of Environment, Faculty of Biology, Jenderal Soedirman University. This study has received an ethical clearance from the Health Research Ethics Commission, Faculty of Health Sciences, Jenderal Soedirman University with number 1123/EC/KEPK/2023. Two 8-week-old male BALB/c mice were obtained from the Faculty of Pharmacy, Universitas Muhamadiyah Purwokerto. Reagents and materials for the in vitro experiment included Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma MCLS), fetal bovine serum (FBS) of non-USA origin sterile-filtered suitable for cell culture (100 mL, Sigma MCLS), amphotericin B solution (250 µg/mL, Elabscience), penicillin-streptomycin solution (100x, Elabscience), latex beads (Sigma), Dulbecco's phosphate buffer saline (DPBS) (Elabscience), phosphate buffer saline (PBS), phytohemagglutinin (PHA)(Gibco), stopper sodium dodecyl sulfate (SDS) (Merck), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Biobasic). All other reagents and chemicals used were of the purest commercial grade available.

Methods

Preparation of the *Z. ottensii* rhizome extract

The maceration extraction method was used to obtain the extract from *Z. ottensii* rhizome. Initially, 2 kg of *Z. ottensii* rhizome simplicia powder was soaked in 12 L of 96% ethanol (1:6) for 24 hours at room temperature. The mixture was then filtered to collect the filtrate. The remaining precipitate was subjected to two additional rounds of maceration using an identical quantity of 96% ethanol each time. The combined filtrates were concentrated using a vacuum rotary evaporator followed by a water bath until a thick extract was obtained. This thick extract was then weighed and underwent further separation for subsequent analysis.

Phytochemical screening with thin layer chromatography (TLC)

The *Z. ottensii* extract was applied to a GF_{254} silica gel plate, which served as the stationary phase. The plate was then placed in a chamber containing a mobile phase mixture of *n*-hexane and ethyl acetate in an 8:2 ratio. After elution process, the plate was removed from the chamber and allowed to air dry until the eluting liquid evaporated. The plate was subsequently sprayed with stain reagents—citroboric for flavonoids and Liebermann-Burchard for terpenoids. The plate was heated at 110°C for 10 minutes. The stains formed were observed under visible light, UV 254, and UV 366 nm.

Immunomodulatory effect on mouse peritoneal macrophages

Two 8-week-old male BALB/c mice were housed with a pelleted basal diet (BR-1) and water ad libitum in an animal room under a 12-hour light/dark cycle at a temperature of 22°C (± 3°C) and a humidity of 60%. The mice were sacrificed by cervical dislocation. To isolate macrophages, 10 mL RPMI 1640 medium was injected into the peritoneal cavity of each mouse. The peritoneal fluid was then centrifuged at 2000 rpm and 4°C for 10 minutes. After the supernatant was removed, the precipitate containing macrophage was resuspended in a medium containing 10% FBS, 2% penicillin and 2% streptomycin, 5% fungizone, and 30% RPMI 1640. The density of macrophages was adjusted to 2,5 x 10^6 cells/mL.

For the phagocytotic activity assay, macrophages were cultured in a 24-well culture plate with coverslips, with 1 mL of cell suspension per well, and incubated at 37°C for 24 hours. The wells were then washed with 1 mL of RPMI 1640 medium and filled with varying concentration of *Z. ottensii* extract. After a one-hour incubation at 37°C, 50 µL of latex beads were added to each well, and the plate was incubated for another hour at 37°C. The wells were washed three times with 1 mL of PBS, fixed with methanol, and stained with 20% Giemsa.

The coverslips were examined under a binocular microscope to assess phagocytotic macrophage activity, calculating both the phagocytotic index and capacity. The phagocytotic index is calculated as the ratio of latex bead particles phagocytosed by activated macrophages, while the phagocytotic capacity was calculated as the ratio of activated macrophages capable of consuming latex particles to 100 macrophages counted.

Immunomodulator effect on lymphocyte proliferation

Mice were sacrificed by cervical dislocation, and their spleens were removed. The lymphocytes were suspended by injecting 10 mL of RPMI 1640

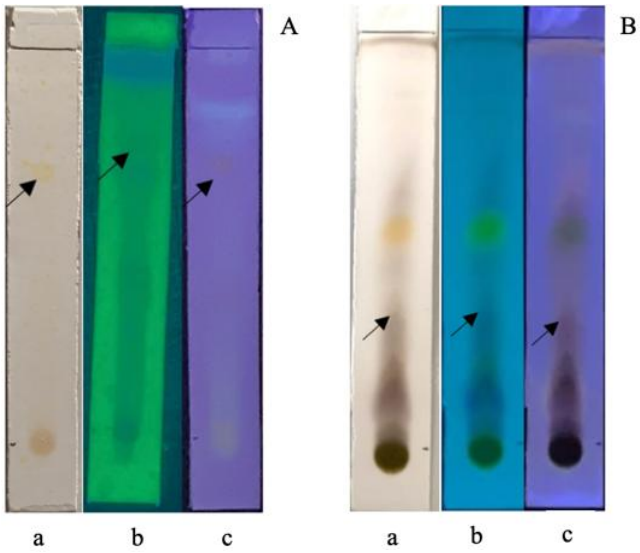

Figure 1. The TLC profiles of *Z. ottensii* extract. (A) Flavonids observed under (a) visible light, (b) UV 254 nm, (c) UV 366 nm. (B) Terpenoids observed under (a) visible light, (b) UV 254 nm, (c) UV 366 nm

solution into the spleen. The suspension was centrifuged at 2000 rpm and 4°C for 10 minutes. The resulting pellet was resuspended in RPMI 1640 and subjected to hemolysis twice using a hemolysis buffer (155 mM NH_4Cl, 15 mM $NaHCO_3$, 1 mM EDTA, pH 7.3), followed by centrifugation at 2000 rpm at 4°C for 10 minutes. After removing the supernatant, the precipitate containing lymphocyte was collected and inoculated into a medium containing FBS 10%, penicillin/streptomycin 2%, fungizone/ amphotericin B 0.5 %, and RPMI 1640. The density of lymphocytes was adjusted to 1,5 $x10^6$ cells/mL.

The cells were cultured in 96-well culture plates with 100 µL of culture medium per well and incubated at 37°C for 24 hours. Wells containing different concentrations of *Z. ottensii* extract were then added, and the plate was incubated for 48 hours at 37°C. Cell viability was measured using the MTT assay and read by an ELISA Reader at 595 nm.

Statistical analysis

Each experiment was conducted in triplicate. Data were recorded as mean ± SEM. Statistical significance across different groups was essessed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software.

RESULTS

Extract and characterization

The rhizome sample used was identified as *Zingiber ottensii* Val. from the Zingiberaceae family. The maceration extraction method, which is simple and preserves volatile compounds (Asworo & Widwiastuti, 2023), was employed. The resulting extract was a thick, brown paste weighing 90 grams with a 9% yield. Qualitative analysis using TLC was performed to identify the compounds present in the *Z. ottensii* extract. TLC results, observed under visible light, UV 254, and UV 366 nm, showed that the *Z. ottensii* extract tested positive for flavonoids and terpenoids. This was indicated by a yellow stain after spraying with citroboric for flavonoids and a purple-red stain for terpenoids after spraying with Liebermann-Burchard (Figure 1).

Immunomodulatory activity of *Z. ottensii* extract

The immunomodulatory influence of *Z. ottensii* extract was examined on both the innate and adaptive immune response. The phagocytotic activity of macrophages was evaluated using a phagocytotic assay. The terms "phagocytotic capacity" and "phagocytotic index" refer to the macrophages' ability to ingest and eliminate foreign objects. Macrophages, isolated from mice's peritonial cavities, undergo phagocytosis when latex beads adhere to them. Latex beads, which have a diameter of about 0.8 µm resembling

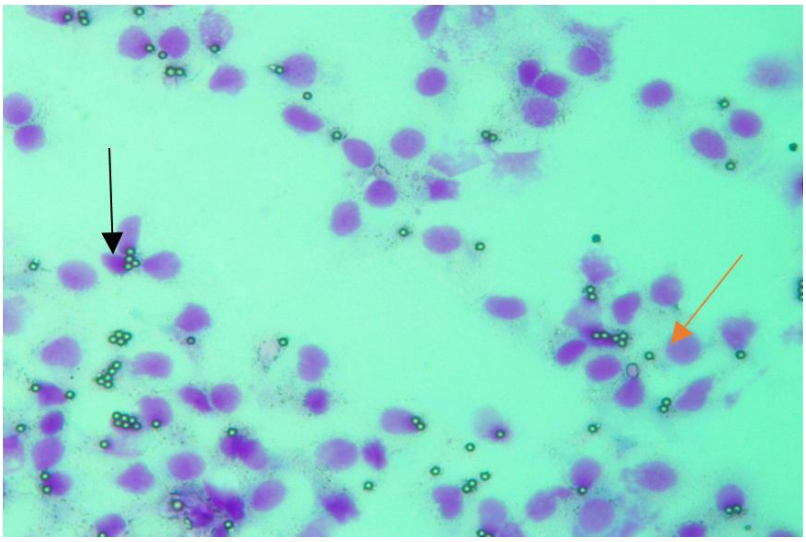

Figure 2. The phagocytotic activity of macrophages in the treatment group. Macrophages were stained with 20% Giemsa, observing at 400x magnification. Active macrophages are visible as purple cells (indicated by black arrow). Latex beads are indicated by red arrow.

the size of bacteria (Ueno et al., 2021), were used as the antigen. Macrophages recognize these beads through surface receptors, such as macrophage receptor with collagenous structure (MARCO) (Cervantes et al., 2014). Staining with Giemsa made the phagocytosed latex beads appear purplish, distinguishing them from non-phagocytosed ones (Figure 2).

The *Z. ottensii* extract at concentration 25, 50, and 100 µg/mL enhanced the phagocytotic capacity of mouse peritoneal macrophages compared to control cells, but the increase was not significantly different (Figure 3). Regarding the phagocytotic index, the *Z. ottensii* extract at 25 and 50 µg/mL significantly increased the index, whereas a higher concentration (100 µg/mL) did not (Figure 4). The 50 µg/mL concentration of *Z. ottensii* extract strongly enhanced the phagocytotic index.

The immunomodulatory effect on the adaptive immune response was assessed by the proliferation lymphocytes in the presence of *Z.ottensii* extract. The ability of *Z.ottensii* extract to stimulate lymphocyte proliferation was measured using the MTT assay. Higher absorbance values indicated higher lymphocyte stimulation index value. The 250 µg/mL of *Z.ottensii* extract did not affect lymphocyte proliferation. However, the extract at concentrations of 15.625, 31.25, 62.5, and 125 µg/mL enhanced lymphocyte proliferation. The 125 µg/mL concentration demonstrated the most significant enhancement in lymphocyte proliferation (Figure 5).

DISCUSSION

Reducing the risk of chronic illnesses involves immune system activation. The immune response can be divided into the innate immune response and the adaptive immune response. Although the adaptive immune response takes time to manifest after infection, it offers a highly targeted defense against pathogens. Within the innate immune system, macrophages perform several vital functions, whereas lymphocytes play an important role in adaptive immune response.

The immunomodulatory effects of *Z. ottensii* rhizome on the immune response were examined in this study. Our findings demonstrated that *Z. ottensii* extract enhanced the phagocytotic activity of mouse peritoneal macrophages compared to control cells. While the extract did not significantly affect phagocytotic capacity, there was a noticeable increase. Specifically, a 50 µg/mL concentration of *Z. ottensii* extract strongly increased the phagocytotic index. Additionally, a 125 µg/mL concentration of the extract markedly stimulated lymphocyte proliferation.

Several rhizomes in the Zingiberaceae family exhibit immunomodulatory potential. For instance, *Zingiber officinale* can stimulate macrophage phagocytosis through immunostimulant activity, while *Zingiber cassumunar* Roxb can suppress macrophage phagocytosis through immunosuppressive activity (Mahfudh et al., 2020). The ethyl acetate fraction of *Z. cassumunar* Roxb rhizomes can boost the expression of IL-10 and IL-14, whereas the

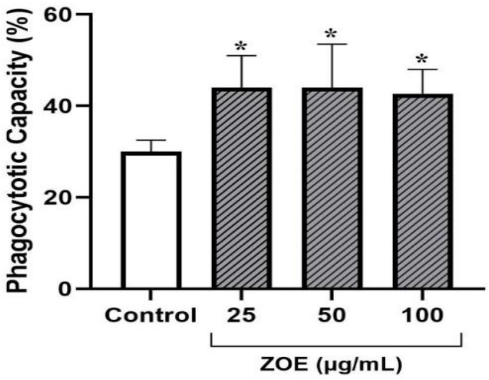

Figure 3. Effect of *Zingiber ottensii* extract (ZOE) on the phagocytotic capacity of mouse peritoneal macrophages. Cells were treated with ZOE at concentrations of 25, 50, and 100 µg/mL and cultured for 1 h. After cultivation, cultures were stained with 20% Giemsa and macrophages were observed. Each result is represented as the mean ± SEM of three independent measurements. Significant differences compared with the control are represented as *$p < 0.05$.

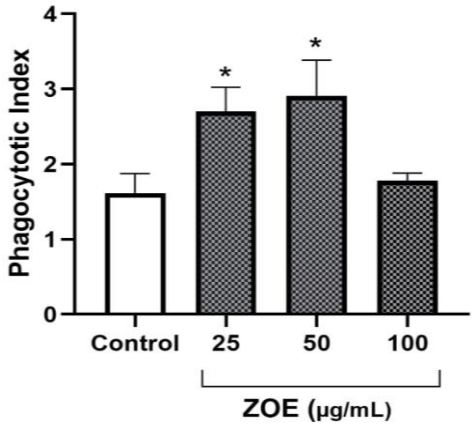

Figure 4. Effect of *Zingiber ottensii* extract (ZOE) on the phagocytotic index of mouse peritoneal macrophages. Cells were treated with the ZOE at concentrations of 25, 50, and 100 µg/mL cultured for 1 h. After cultivation, cultures were stained with 20% Giemsa and macrophages were observed. Each result is represented as the mean ± SEM of three independent measurements. Significant differences compared with the control are represented as *$p < 0.05$.

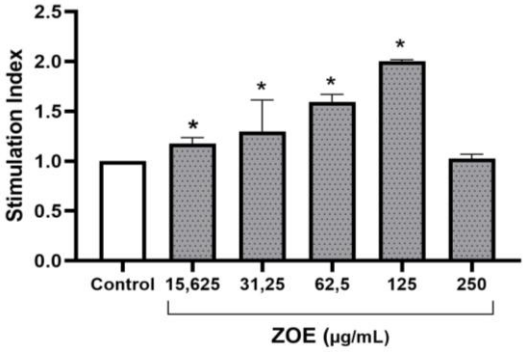

Figure 5. Effect of *Zingiber ottensii* extract (ZOE) on the stimulation index of lymphocyte proliferation. Cells were treated with the ZOE at concentrations ranging from 15.625 - 250 µg/mL and cultured for 48 hours. After cultivation, cell viability was measured using the MTT assay and read by ELISA Reader at 595 nm. Each result is represented as the mean ± SEM of three independent measurements. Significant differences compared with the control are represented as *$p < 0.05$.

n-hexane fraction inhibits lymphocyte proliferation and macrophage phagocytic activity (Nurkhasanah, et al., 2019a; Nurkhasanah et al., 2019b). Similarly, the ethanol extract of *Z. officinale* enhance macrophage and lymphocyte activity (Masniah et al. 2021). The essential oils of *Z. ottensii* have demonstrated anti-inflammatory and anti-cancer properties (Panyajai et al., 2022; Thitinarongwate et al., 2022).

Z. ottensii has also shown activity in increasing the proliferation and differentiation of CD8+ T lymphocytes (cytotoxic T cells/CTL) (Panyajai et al., 2022; Ruttanapattanakul et al., 2021). Increased lymphocyte proliferation activity leads to an increase in both CTL and CD4+ T lymphocytes (helper T cells) (Abbas *et al*., 2022). These cells aid the B lymphocyte response in producing antibody that can bind to specific antigens. Another study indicated that *Z. officinale* extract at 50, 100, and 200 µg/mL could stimulate T and B cell proliferation (Yücel *et al*., 2022). Additionally, *Z. ottensii* extract has been shown to inhibit COX-2 production (Koontongkaew et al., 2013; Thitinarongwate et al., 2022). The extracts has a strong cytotoxic effect with an IC_{50} of 60 µg/mL compared to other plants (Suprihatin et al., 2020). Therefore, *Z. ottensii* extract could enhance the immune system by increasing phagocytotic index and lymphocyte proliferation, suggesting its potential as an adjuvant for immunopreventive drugs, warranting further *in vivo* studies.

CONCLUSION

Zingiber ottensii extract was confirmed to contain flavonoids and terpenoids. *Zingiber ottensii* extract demonstrated the ability to modify lymphocyte proliferation and phagocytotic activity. By increasing the phagocytosis index and lymphocyte proliferation stimulation index, the *Z. ottensii* extract can enhance macrophage phagocytic activity. Thus, the rhizome of *Z. ottensii* can improve human health by preventing disorders linked to the immune system.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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