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Immunomodulatory Activity of Robusta Green Coffee Extract on Macrophage Phagocytosis and Lymphocyte Proliferation in Vitro

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Abstract

Robusta green coffee contains a large quantity and variety of polyphenols and flavonoids which are known to have antioxidant and anti-inflammatory properties. This research to investigate the immunomodulatory activity of Robusta green coffee extract on macrophage activity stimulated with lipopolysaccharide (LPS) and lymphocyte proliferation. Macrophages were isolated from mice peritoneal tissue, and Lymphocytes were isolated from lymph organs. The immunomodulatory activity on macrophages was evaluated based on Active Phagocytes Cells (APC) and the proliferation of lymphocytes was evaluated by the MTT Assay which was previously given extracts with varying concentrations (20 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 µg/ml), then the data were analyzed by ANOVA (α=0.05). The aqueous extracts of Robusta green coffee significantly affects macrophage activity and lymphocyte proliferation in vitro. The extract was decrease the percentage of phagocytosis (PP) macrophage stimulated with LPS, however there was increase phagocytosis capacity (PC) and lymphocyte proliferation stimulation index is < 3, illustrating the extract has a low immunomodulatory effect on lymphocyte proliferation. Based on these results, Robusta green coffee extract holds potential as an immunomodulatory agent, affecting against macrophage phagocytosis and lymphocyte proliferation.

1. INTRODUCTION

The interest of Indonesian population in natural immunomodulators is currently on the rise [1]. This trend can be observed during and after the Covid-19 pandemic, where the consumption and knowledge of the Indonesian population regarding natural immunomodulators increased from 58% to 75%, in alignment with a widespread public awareness campaign [2].

Many studies have demonstrated that natural immunomodulators have therapeutic effects on the immune system. Natural immunomodulators accelerat the healing process of infections and reduc negative effects in infected patients. This is evidenced by activities such as phagocytosis activity, lymphocyte proliferation, and the secretion of pro-inflammatory cytokines. Natural Immunomodulators are less toxic, than do not show significant side effects [3,4].

One of the plants known to have the potential as a natural immunomodulator is Robusta green coffee (*Coffea canephora*). Coffee is known to be rich in sources of chemical compounds with strong antioxidant and Anti inflamation activity [5]. This study focuses on how Robusta green coffee extract can modulate immune cells specifically through macrophage phagocytosis and lymphocyte proliferation.

Based on the screening of bioactive compounds in green coffee beans (*Coffea canephora*), nearly 83% are polyphenolic compounds with a chlorogenic acid content of 14.4%, and the caffeine content ranges around 2.38% [6]. In addition, other bioactive compounds such as flavonoids, sterols, cafestol, kahweol, trigonelline and ceramides are also present in green coffee beans [7].

In previous research, chlorogenic acid content has been shown to independently reduce the production of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interferon (IFN-Y), and interleukins (IL-1β, IL-6). Meanwhile, caffeine has also been tested and found to reduce the expression of pro-inflammatory genes, including those induced by nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), thereby affecting the production of interleukins (IL-3, IL-6, and IL-12), and stimulated the proliferation of B cells, Th1, and Th2 cells [8-9].

Macrophages function primarily in the early innate immune response as part of the host defense mechanism. Meanwhile, lymphocyte cells function to maintain specific immune responses including cellular (associated with T cells) and humoral (associated with antibodies or B cells) immune responses [10]. Therefore, the aim of this research is to investigate the potential immunomodulatory activity of Robusta green coffee on the phagocytosis of peritoneal macrophage cells induced by inflammation with LPS and on lymphocyte cell proliferation.

2. MATERIALS AND METHODS

Materials and Samples

The research material used was unroasted Robusta coffee beans (*Coffea canephora*), which were obtained from a coffee plantation in Pasuruan, East Java. Subsequently, taxonomic identification was conducted at the Biology Laboratory of the Faculty of Science and Technology at Maulana Malik Ibrahim State Islamic University.

The test animals used were 2 male Balb/c mice (*Mus musculus*) obtained from the White Rat Breeding Facility in Malang. The selected experimental animals were males aged 6-8 weeks and free from pathogens. The materials used included RPMI medium, Phosphate Buffer Saline (PBS) without calcium and magnesium or physiological NaCl (9.8%), and complete FBS 10% medium.

Robusta Green Coffee Extraction

The Robusta coffee beans (*Coffea canephora*) were ground into powder using a grinder. A total of 100 mg of finely ground green coffee powder was extracted with 600 ml of distilled water (at a ratio of 1:6) using the Infuse water method at a temperature of 70-75°C for 2 hours [11]. The resulting infusion was

then filtered using flannel cloth and filter paper to separate the residue from the filtrate. The residue was subsequently re-infused with water at the same temperature and duration. The filtrate obtained was then dried using freeze-drying to obtain dry extract powder. The green coffee extract was prepared into solutions with various concentrations (30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, and 70 µg/ml).

Isolation of Macrophage and Lymphocyte Cells

Macrophages were isolated from the peritoneal tissue of mice that had been euthanized by cervical dislocation. The abdominal area was sterilized with 70% alcohol, the outer skin was opened, and 10 ml of cold RPMI media was injected into the peritoneal cavity using an 18G syringe. After 3 minutes, the media in the peritoneum was resuspended and then transferred into a 15 ml tube [12].

Lymphocytes were isolated from the spleen tissue, which was minced with a glass object and a syringe in 5 ml of PBS media. The mixture was then filtered through a 70 µm Cell Strainer and transferred into a 15 ml tube. The fluid was centrifuged at 1200 rpm at 4°C. The supernatant was discarded, and 3 ml of media with 5% FBS and Tris Buffer Solution (TBS) to lyse red blood cells were added to the pellet. It was then centrifuged again at the same speed and temperature. The supernatant was discarded, and the pellet containing macrophages was resuspended in 3 ml of media with 10% FBS [13]. The peritoneal macrophages and lymphocytes can now be used for further experiments or treatments.

Macrophage Phagocytic Activity Test

Macrophage cells, 2.5x10^5 cells/well, were placed in 3 ml of RPMI media with 10% FBS at 37°C with 5% CO2 for 24 hours. After the macrophages adhered to the well bottom, they were treated with various concentrations of extract for 24 hours of incubation. Subsequently, the cells were exposed to 1 µg/ml of LPS and incubated for an additional 1 until 2 hours at 37°C with 5% CO2. Following the LPS treatment, the material was washed with RPMI media, and 150 µl of latex beads, previously resuspended in PBS with 10% FBS at a density of 2.5 x 10^7 latex/ml, were added. Incubation was continued at 37°C with 5% CO2 for 1 hour.

After incubation, the plates were washed with PBS three times to remove non-phagocytosed latex beads and left to air dry at room temperature. Subsequently, they were fixed with absolute methanol for 30 seconds and allowed to dry at room temperature. The cells were then stained with 20% Giemsa stain for 20-30 minutes [14].

$$PP = \frac{\text{Number of actively phagocytic macrophages}}{\text{100 Macrohage cells}} \times 100\%$$

$$PC = \frac{\text{Number of latex beads that have been phagocytosed.}}{\text{50 Macrohage cells}}$$

note.
PP: Phagocytosis Percentage
PC: Phagocytosis Capacity

The plates were washed with sterile distilled water three times and dried at room temperature. The number of macrophages that phagocytosed latex beads and the number of latex beads phagocytosed by active macrophages were then counted using an inverted microscope or a light microscope at 200x magnification.

Lymphocyte Proliferation Test

Suspension of lymphocyte cells (100µL) was added to a 96-multiwell plate. The plate was then incubated in a CO2 incubator for 24 hours at 37°C. Samples were subsequently treated with a series of concentrations: 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, and 70 µg/ml, with three replicates each, and incubated for 48 hours.

Afterward, 10 µL of MTT was added to each well and incubated for 4 hours. Then, 100 µL of a stop solution (10% SDS in 0.01 N HCl) was added to each well to terminate the reaction. Absorbance was read using an ELISA reader at 490-550 nm, and lymphocyte

proliferation was calculated using the stimulation index [13].

$$SI = \frac{\text{Absorbance Value (Sample - Media)}}{\text{Absorbance Value (Normal - Media)}}$$

note.
SI: Stimulasi Indeks

Data analysis

The phagocytic activity of macrophages and lymphocyte proliferation were statistically analyzed using SPSS to determine if there were significant differences among the treatment groups. The data were analyzed using a One-Way ANOVA with a confidence level of 95%. Subsequently, post hoc Duncan's tests were conducted to assess the differences in the effects of each treatment.

3. RESULTS and DISCUSSION

This study focused on how Robusta green coffee extract could modulate immune cells, specifically through macrophage phagocytosis and lymphocyte proliferation. Robusta green coffee was extracted using a water solvent and infusion method. To remove the water content, Robusta green coffee extract was dried using freeze-drying until it became a powdered extract. This drying method aimed to prevent damage to the active compounds contained in the extract and extended its utility without altering the active compounds within.

Effect of Robusta Green Coffee Extract on Macrophages Phagocytosis Activity

Macrophages were cells about 9-12 µm in size with a relatively large nucleus and often had many branches or pseudopodia (Figure 1a). To determine the immunomodulatory effect of Robusta green coffee extract on macrophage activity, a phagocytosis test was conducted in vitro. The parameters used were Active Phagocytic Cells (APC), which referred to the percentage of macrophage cells (PP) that phagocytized latex particles out of 100 macrophage cells, and phagocytic capacity (PC), which referred to the number of latex particles phagocytosed [14]. Phagocytic activity was indicated by latex particles contained in phagosomes in the macrophage cytoplasm (Figure 1b).

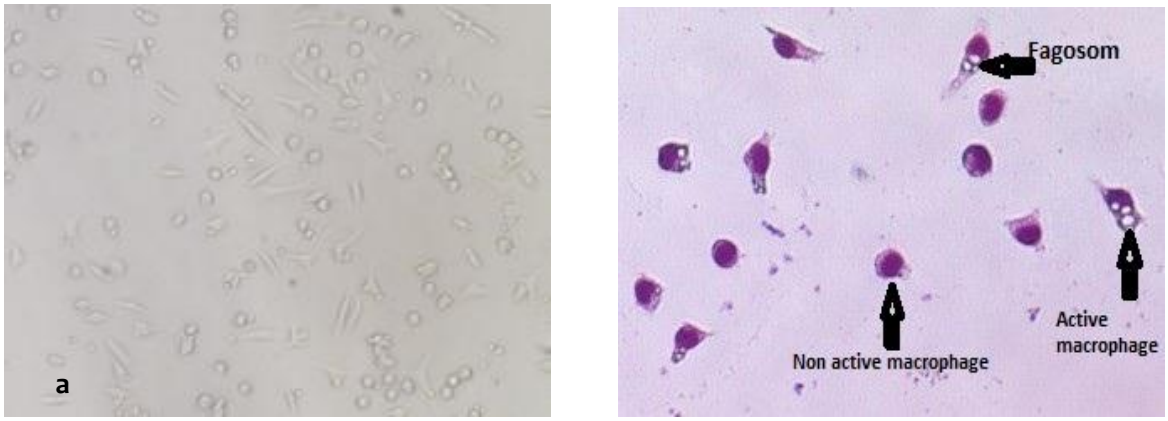

Figure 1. (**a**) Unstained macrophage cells; macrophage cells appear relatively large and have numerous branches or pseudospodia. (**b**) Macrophage cells are stained with Giemsa 20%. After induction with lipopolysaccharide (LPS) at a concentration of 1 µg/ml, phagocytic activity can be seen in macrophages, displaying phagosomes in the cytoplasmic area. In contrast, macrophages not actively engaged in phagocytosis do not exhibit the presence of phagosomes.

This research involved five treatment variations of the extract with concentrations of 30, 40, 50, 60, 70 µg/ml, as well as normal control and LPS (1µg/ml) groups. In the extract treatment groups, macrophage cells were induced with LPS to induce an inflammatory

response. The results of the immunomodulatory effect test of Robusta green coffee extract on macrophage phagocytosis activity in vitro (Figure 2a) showed that the treatment groups were able to reduce the percentage of macrophage phagocytosis after LPS induction.

The percentage of macrophage phagocytosis in the extract treatment groups of 30, 40, 50, 60, and 70 µg/ml was 81.0%, 82.75%, 74.75%, 71.0%, and 79.00%, respectively, compared to the macrophage group induced with LPS, which reached 87.50%. There was a decrease in the percentage of macrophage phagocytosis in the treatment groups with doses of 50 and 60 µg/ml by 16.50% and 12.75%, while at doses of 30, 40, and 70 µg/ml, the decrease in the percentage of phagocytosis was 6.5%, 4.75%, and 8.50%, respectively.

Based on the results of the statistical analysis using Anova and Duncan's post-hoc test, it is concluded that the extract significantly ($P<0.05$) has an effect on reducing the percentage of macrophage phagocytosis (PP) that undergoes inflammation due to LPS induction. The dose of 60 µg/ml showed a significant decrease in PP by 16.50%, which differed significantly from the group induced only by LPS. Meanwhile, PP of macrophages at other doses did not show a significant difference compared to the LPS control and normal groups.

The treatment with LPS was found to have increased the macrophage phagocytosis percentage (PP) by 7.45% compared to the normal group. This indicated that LPS induction in macrophages could activate phagocytosis against latex particles, reflecting an inflammatory condition. The decrease in PP of macrophages after extract treatment indicated an immunomodulatory effect, suppressing the phagocytic activity of inflamed macrophages.

On the PC parameter, the immunomodulatory effect of Robusta green coffee extract showed an increase in the phagocytic capacity (PC) of inflamed macrophages after LPS induction (Figure 2b). At extract doses of 30, 40, 50, 60, and 70 µg/ml, the average PC of macrophages reached 106.50 latex, 113.00 latex, 124.70 latex, 113.00 latex, and 123.00 latex, respectively. This data indicated an increase compared to the LPS group, which only achieved a PC of 105.25 latex, not significantly different from the normal group, which reached 103.25 latex.

The results of the statistical analysis using ANOVA and LSD post-hoc test concluded that the extract significantly ($P<0.05$) had an effect on the phagocytic capacity (PC) of inflamed macrophages due to LPS induction. The doses of 60 µg/ml and 70 µg/ml showed a significant increase in PC of macrophages compared to the group induced only by LPS and the normal group. Meanwhile, the PC of macrophages at other doses did not show a significant difference compared to the LPS control and normal groups

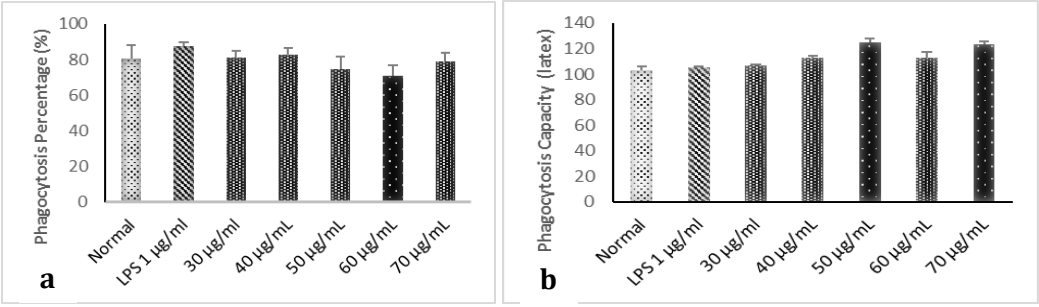

Figure 2. Profile of macrophage phagocytosis activity following treatment with Robusta green coffee extract indicates that the extract has an effect on the phagocytosis of inflamed macrophages after LPS induction. (**a**) The immunomodulatory activity of the extract suppress the percentage of phagocytosis, (**b**) while the extract stimulate the phagocytic capacity.

Immunomodulatory Activity on Lymphocyte Proliferation

The immunomodulatory activity of Robusta green coffee extract against lymphocyte proliferation was tested using the MTT Assay method. The MTT Assay measures light absorption by formazan crystals formed as a product of MTT reduction into insoluble formazan, catalyzed by the enzyme succinate dehydrogenase. This enzyme is produced from the mitochondria

of living cells, indicating that formazan crystals can only form in living cells. The number of live cells is positively correlated with formazan crystals and absorbance values.

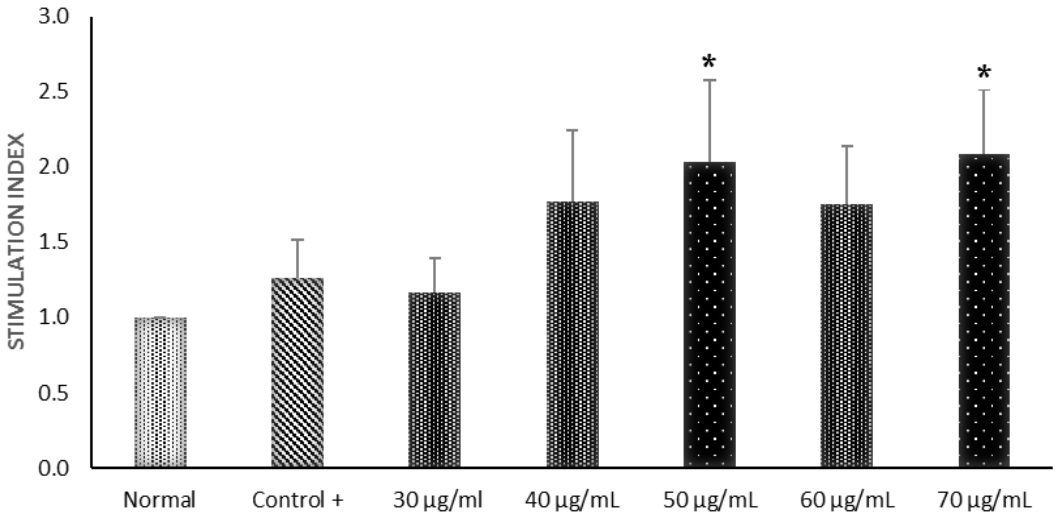

Figure 3. Profile of lymphocyte proliferation stimulation index. The immunomodulatory activity of Robusta green coffee extract shows an increased lymphocyte proliferation

Based on the research results, Robusta green coffee extract showed an increase in lymphocyte proliferation in vitro (Figure 3). In the treatment groups with doses of 30, 40, 50, 60, and 70 µg/ml, the Stimulation Index (SI) of lymphocyte proliferation was observed to be 1.2, 1.8, 2.0, 1.8, and 2.1 SI, respectively. The positive control treatment group (X-immunomodulator) showed an increase in the SI of lymphocyte proliferation by 1.3, compared to the normal group, which had an SI of lymphocyte proliferation of 1.0 SI.

The results of the statistical analysis using Anova and the subsequent DMRT post-hoc test concluded that the extract significantly ($P<0.05$) influenced lymphocyte proliferation, as indicated by the increased Stimulation Index (SI). Doses of 40, 50, 60, and 70 µg/ml showed a significant increase in SI of lymphocyte proliferation compared to the normal group.

Meanwhile, the dose of 30 µg/ml did not show a significant difference compared to the normal group. Based on these findings, the immunomodulatory effect of Robusta green coffee extract could stimulate lymphocyte proliferation in vitro. Observations of the immunomodulatory effect on lymphocyte proliferation under an inverted microscope are depicted in Figure 4.

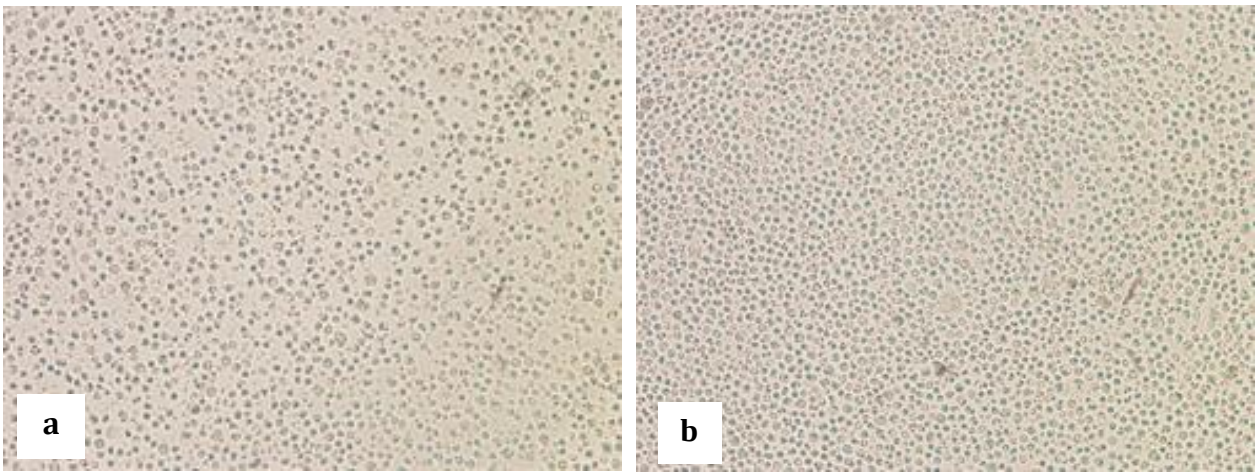

Figure 4. Observing lymphocyte proliferation through an inverted microscope. Prior to treatment with Robusta green coffee extract, the culture plate exhibits numerous gaps, indicating non-confluent cells (**a**). After five days of extract treatment, the gaps on the plate start to close, and cells have now reached confluence, signifying lymphocyte proliferation (**b**). The immunomodulatory activity of the extract can impact lymphocyte proliferation

4. DISCUSSION

Robusta green coffee is a processed form of coffee beans that has not undergone roasting. Green coffee contains more phytochemical content compared to roasted coffee, including chlorogenic acid, caffeine, flavonoids, saponins, trigonelline, and the diterpene kahweol. This is because the roasting process can transform phenolic compounds into volatile compounds and melanoidins, which contribute to the coffee flavor. Consequently, the antioxidant and anti-inflammatory properties of Robusta green coffee are higher, and its impact on the immune response experiencing inflammation is more optimal [5,7].

Based on the research results, Robusta green coffee extract significantly influences the phagocytosis activity of inflamed macrophages induced by LPS and affects lymphocyte proliferation in vitro. The extract can suppress the percentage of phagocytosis (PP) in inflamed macrophages (Figure 2a). On the other hand, the extract can enhance the phagocytic capacity (PC) of macrophages against latex particles (Figure 2b) and increase lymphocyte proliferation (Figure 3).

This is consistent with previous research findings that concluded Robusta green coffee extract indeed influences the phagocytosis activity of macrophages by increasing both the percentage and capacity of phagocytosis [14]. In the treatment of various Robusta coffee brews, it was also proven to enhance the phagocytic capacity of macrophages previously induced by Bacillus cereus [15]. The immunomodulatory activity of the active compounds in Robusta coffee significantly affects the proliferation of both B and T cells [5,16].

The increase in phagocytic capacity (PC) and the decrease in the percentage of phagocytosis (PP) in LPS-induced macrophages perhaps attributed to the bioactive compounds in Robusta green coffee extract, which exhibit anti-inflammatory and antioxidant activities. Anti-inflammatory components such as chlorogenic acid and caffeine can suppress the production of proinflammatory cytokines like TNF-α, IL-1β, and IL-6 in LPS-stimulated macrophages (RAW264.7) [8,9]. The reduction in cytokines

TNF-α, IL-1β, and IL-6, it's impact the phagocytic activity of macrophages by suppressing the number of actively phagocytosing macrophages [17]. Caffeine also influences the production of IFN-γ and IL-2 by lymphocytes and has been shown to maintain oxidative balance in lymphocytes, protecting them from oxidative damage caused by H_2O_2 [18,19].

Robusta green coffee extract also exhibits high antioxidant activity. Chlorogenic acid and caffeine are proven to stabilize Reactive Oxygen Species (ROS) generated during the phagocytic activity, reduce the production of nitric oxide (NO) and cyclooxygenase-2 (COX-2) in infected macrophages and lymphocytes. Meanwhile, flavonoids in green coffee are known to enhance the opsonization of phagocytic cells [19-22].

These actions can stabilize oxidative stress in cells, preventing cell damage, maintaining membrane permeability, and ensuring cellular functions remain stable. As a result, they enhance the macrophage's capacity to phagocytose foreign particles and promote lymphocyte proliferation [19,23]. Based on these factors, Robusta green coffee extract significantly influences the innate immune response of inflamed macrophages and the adaptive immune response by increasing lymphocyte proliferation.

5. CONCLUSION

Study could be concluded that Robusta green coffee extract with a water solvent had immunomodulatory activity that affected the phagocytic activity of LPS-induced macrophages and influenced lymphocyte proliferation in vitro. The immunomodulatory activity of the extract reduced the percentage of macrophage phagocytosis, while on the other hand, it increased the phagocytic capacity of macrophages and enhanced lymphocyte proliferation. The dose of 60 showed the highest suppression of phagocytic percentage, while doses of 50 and 70 showed the highest increase in macrophage phagocytic capacity and lymphocyte proliferation, which were not significantly different.

6. ACKNOWLEDGMENT

In the future, research is expected to investigate the immunomodulatory effects of the extract on proinflammatory cytokines, including TNF, IL-1, and IFN. Further studies will be conducted on the toxicity of the extract. Additionally, in vivo will be involved to validate the results of in vitro research

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