

The Effect of *Moringa oleifera* Leaf Extract on Phagocytosis Activity of Macrophages, ros, no, Ifn- γ , and Il-10 on PbmC in Adult Pulmonary Tuberculosis Patients Induced by Esat-6 *in-vitro*

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ABSTRACT

Introduction: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB). MTB can inhibit phagolysosomal fusion, so it's difficult to eliminate, and the BCG vaccine didn't contain ESAT-6/CFP-10, which caused therapy and immunization couldn't be optimal in eradicating TB. An immunomodulator is needed to improve the immune system, especially from natural ingredients. A *Moringa oleifera* plant is known to have immunomodulatory properties because of its nutrients, flavonoids, polyphenols, saponins, alkaloids, and steroids.

Material and Methods: This study aims to determine the effect of the *M. oleifera* leaf extract (MLE) on the phagocytic activity of macrophages, ros, no, Ifn- γ , and IL-10 induced by ESAT-6 in the Peripheral Blood Mononuclear Culture (PBMC) pulmonary TB patients. The research was carried out on PBMC from the blood of six pulmonary TB patients with a post-test-only control group design. The samples were divided into three groups: negative control group, positive control group (induced with ESAT-6), and intervention groups (induced with ESAT-6 and MLE at doses of 1 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 25 $\mu\text{g/mL}$).

Results: MLE with a dose of 1 $\mu\text{g/mL}$ significantly increased macrophages' phagocytic activity, ros/no, Ifn- γ , and decreased IL-10 levels ($p < 0.05$) as compared to the control group, whereas at a dose of $\geq 2.5 \mu\text{g/mL}$, it decreased macrophages phagocytic activity, ros/no, Ifn- γ , and increased IL-10 levels.

Discussion: These indicate that MLE at a low dose had immunostimulant activity, but at a higher dose showed immunosuppressive and anti-inflammatory activity.

Keywords: ESAT-6, Ifn- γ , IL-10, Macrophage, *Moringa oleifera*, Tuberculosis.

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INTRODUCTION

TB disease is one of the top 10 causes of death in the world after HIV/AIDS. By the end of 2019, an estimated ten million people will suffer from TB disease.¹ MTB is an intracellular bacteria that lives and reproduces in macrophages cells, can withstand lysosomal enzymes, and can inhibit phagolysosomal fusion, making it difficult to eliminate. In a dormant state, they can hide in cells for long periods, without being tracked by the immune system, making the global eradication of MTB very difficult.² Prevention efforts in the form of immunization are the most effective intervention in controlling the disease. The disadvantage of BCG is the loss of the genome region RD1 that expresses the Esx-1 type VII secretion system, causing a non-maximal protective effect. Esx-1 encodes Esx-A (Early Secretory Antigenic target-6/ESAT-6) and Esx-B (Culture Filtrate Protein-10/CFP-10), which are potential vaccine candidates that are still being researched and developed, and are used as vaccine candidates and diagnostic markers of TB disease. ESAT-6 and CFP-10 are also virulence and pathogenicity factors for MTB. Mechanisms of virulence and pathogenicity of ESAT-6 and CFP-10 are still being investigated, but are predicted to

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cause cytolysis of alveolar epithelial cells and macrophages.³ Several studies have begun to focus on the ESAT-6 antigen, due to the high levels of ESAT-6 antibodies found in pleural effusions⁴ and granulomas.⁵ ESAT-6 antibody levels can last up to 1 year compared to CFP-10 in experimental animals infected with MTB.⁶ Several studies using the ESAT-6 antigen *in vitro* on Peripheral Blood Mononuclear cells (PBMC) of TB patients showed an increase in IL-10 levels, while the levels of IFN- γ are still controversial.^{7,8} ESAT-6 is predicted to cause phagosome rupture so that MTB avoids elimination and translocates to the cytosol of macrophages cells.⁹ ESAT-6 is predicted to be a virulence factor and pathogenicity factor of MTB that can inhibit macrophages in phagocytosis activity,³ inhibit the production of ROS/NO in eliminating MTB.^{10,11} ESAT-6 is an antigen that is predicted to induce IFN-, but can inhibit the production of IFN- γ .⁸ ESAT-6 can increase the production of IL-10.^{7,8}

Treatment of TB patients requires close supervision for long-term treatment success, but the occurrence of treatment failure is quite high, which causes an increase in the number of TB patients. Drug-resistant TB is more difficult to treat and increases the cost of TB control programs in endemic countries.¹

Based on these circumstances, efforts are needed to develop new TB therapies. One of the ways to develop therapy is by providing nutrition, as well as improving the immune system, using certain compounds or materials as immunomodulators. It is hoped that immunomodulators can be used as immune restoration for a dysfunctional immune system.¹² Immunomodulators are expected to increase IFN- γ levels, reduce IL-10 levels, stimulate macrophages phagocytosis activity, and can increase the production of reactive oxygen intermediates and reactive nitrogen through the respiratory burst killing mechanism to eliminate MTB.¹³ The lack of immunomodulators is that they have to be given repeatedly to produce cytokines that can improve the immune system, so high availability of immunomodulators is needed so that they can be given repeatedly in the long term, such as immunomodulators derived from natural ingredients.

Moringa Olifera is a plant that is easy to grow in Indonesia, as an alternative herbal plant that can be used as a supplement to improve nutritional status because it has a fairly high micronutrient content. In addition to the low price, this plant can be consumed as a food source that is rich in protein (amino acids), minerals, and vitamins. Many natural bioactive components have been found in the *M. oleifera* plant, including phenolic compounds, flavonoids, tannins, alkaloids, saponins, and steroids.¹⁴

Research on the *M. Olifera* leaves for antihypertensive, antilipidemic, anti-inflammatory, antioxidant, and anticancer properties has been widely carried out. But, the research on their immunomodulatory activity is still not widely done. One study reported that the *M. Olifera* leaves can increase the immune system of white mice injected with cyclophosphamide.¹⁵ The methanol leaf extract given to rats induced with

Pasteurella multocida bacteria can increase immunoglobulin levels, increase the number of neutrophils, and increase phagocytosis index, after previously being induced with cyclophosphamide.¹⁶ Several studies reported that there was an increase in the phagocytosis index in rats after being given the leaf extract.^{16,17} Another researcher reported that there was an increase in CD4 + T cells (T helper) and CD8 + T cells (cytotoxic T lymphocyte cells) with the administration of the leaf extract in rats induced by *Salmonella typhi*.¹⁸

This study was conducted to evaluate the activity of the *M. oleifera* leaf extract (MLE) as an immunomodulator in terms of macrophages phagocytosis activity, macrophages ROS and NO levels, and the ratio of IFN- γ and IL-10 in TB patients induced by ESAT-6 antigen *in vitro*.

MATERIALS AND METHODS

Plant Materials

The leaves of *M. Olifera* were collected from the Blora region of Indonesia. This plant was authenticated in the School of Life Science and Technology, Bandung Institute of Technology, Indonesia. The leaves were washed and air dried out of the direct sunlight (Voucher number: 5090/11.CO2.2./PL/2019).

Extraction of *M. Olifera* Leaves

The dried leaves were powdered and oven-dried at 40°C until the constant weight. The powdered leaves were macerated with 70% of ethanol solvent within 1 x 24 hours at room temperature, and this treatment was repeated three times. The results of this maceration were filtered to separate the extract and the dregs. The extract was then evaporated under lower pressure at the temperature of 40°C for 2 x 24 hours to yield concentrated extracts. The extract was made into preparations of 25, 10, 2.5, and 1.0 $\mu\text{g/mL}$.

Experimental Design

This research is an experimental laboratory study with a post-test-only control design with PBMC in adult active pulmonary TB patients *in vitro*.

Subjects

Subjects used in this study were adult active pulmonary TB patients from a certain national hospital in Bandung, Indonesia who met inclusion criteria. The inclusion criteria for this study were patients with active pulmonary TB aged > 18 years, diagnosed for the first time, had not been able to receive anti-TB oral therapy, and had never been used as study subjects. Exclusion criteria were TB patients with severe malnutrition, liver disease, kidney disorders, heart disease, diabetes, malignancy, and HIV. The diagnosis of TB is made by a specialist in internal medicine based on clinical symptoms, chest x-ray, sputum, and rapid molecular tests. After the diagnosis was established and the patient understood and signed the informed consent, the subject data was recorded in the form, and a blood sample of ± 10 cc from the vein was taken.

Samples

A total of ± 60 cc of blood from 6 patients from Pindad Hospital in Bandung was brought to the Aretha Laboratory in Bandung for research.

Examination of Macrophage's Phagocytic Activity and Measurement of ROS/NO Levels, IFN- γ Levels, and IL-10 Levels

The blood samples were taken from six TB patients in one of the national hospitals in Indonesia and divided into three group treatments consisting of a negative control group NC (without induction with ESAT-6), a positive control group PC (induced with ESAT-6), and the intervention groups P1-P4 (induced with ESAT-6 plus MLE at doses of MLE 1, 2.5, 10, and 25 $\mu\text{g}/\text{mL}$). All the MLE doses and the ESAT-6 dose of 5 $\mu\text{g}/\text{mL}$ were determined based on reference and preliminary studies, which provide the most optimal proliferation of PBMC cultures.

The blood samples were suspended into PBMCs and cultured in RPMI 1640 media (L0500-500, Biowest) for 5 days, then added Phorbol 12-Myristate 13-Acetate/PMA (1544-5, Bio Vision Incorporated), so that monocytes proliferate into macrophages. The 5-day-old PBMC culture was added with 5 $\mu\text{g}/\text{mL}$ ESAT-6 (abx169018, Abxbexa), except for negative control, incubated for 24 hours, followed by the administration of MLE, incubated for 24 hours, and laboratory tests were carried out. In the macrophages phagocytosis activity test, Latex Beads Amine-Modified Polystyrene Fluorescent Blue (L0280, Sigma Aldrich) suspension was added, incubated for 1-2 hours, then counted the number of macrophages cells that phagocytize latex particles. Intracellular ROS levels were measured using DCFDA-Cellular Reaction Oxygen Species Detection Assay Kit reagent (ab113851, Abcam), which was analyzed by flowcytometry (Miltenyi Biotec). Intracellular NO levels were analyzed using The Greiss Method. IFN- γ levels were analyzed using the Human IFN- γ ELISA Kit Reagent (E-EL-H0108, Elabscience). IL-10 levels were analyzed using The Human IL-10 ELISA Kit (430601, BioLegend).

Ethical Considerations

The Research Ethics Committee Universitas Padjadjaran Bandung Indonesia has given its approval to this study (Number: 1499/UN6.KEP/EC/2019).

Time and Place of Study

The study was conducted from October 2019 to December 2020 in the Aretha Laboratory in Bandung, Indonesia.

Data Analysis

Univariate and bivariate analyses were used on the data obtained. The univariate analysis consisted of a frequency distribution (mean, standard deviation), and the Shapiro-Wilk test (normally distributed sample data). Bivariate analysis

conducted was One Way ANOVA test to find out whether there were differences between groups. To find out which groups were significantly different, a Post-Hoc test (Tukey's test) was performed. All statistical calculations were carried out using IBM SPSS 25 Statistical Package.

RESULTS

Macrophages Phagocytic Activity

An examination of the effect of MLE against macrophage phagocytic activity was conducted on blood samples taken from six TB patients, and the results are shown in Table 1 or Figure 1. The addition of ESAT-6 as a pathogenicity factor for MTB on the blood sample in the positive control decreased the phagocytic activity of macrophages significantly as compared to the negative control. In the intervention groups where the blood sample was added with ESAT-6 and MLE at doses of 1, 2.5, 10, and 25 $\mu\text{g}/\text{mL}$, the macrophage phagocytic activity increased according to the reducing doses. All doses significantly increased the macrophage phagocytic activity compared to the positive control ($p < 0.05$) but still lower than the negative control.

Measurement of ROS Levels

The results of ROS level measurement are presented in Table 2 or figure 2. In the positive control group, the addition of ESAT-6 into the blood sample decreased the production of intracellular ROS (percentage of 2.16) significantly different from the negative control (percentage of 3.34). MLE at low doses increased the intracellular ROS level, but as the dose increased, the ROS level decreased. The dose of 25 $\mu\text{g}/\text{mL}$ of MLE showed a significant decrease in the ROS level when compared to the positive control ($p < 0.05$).

Measurement of NO Levels

The measurement of NO levels resulted in data as shown in Table 3 or figure 3. The NO level in the positive control group decreased significantly from the negative control ($p < 0.05$). The administration of MLE at all doses showed a significant NO level increase compared to the positive control ($p < 0.05$).

Measurement of IFN- γ Levels

Table 4 or figure 4 shows that the administration of ESAT-6 on the blood sample in the positive control group decreased IFN- γ levels significantly compared to the negative control ($p < 0.05$). MLE increased the IFN- γ levels and a significant increase was shown by the lowest dose of MLE (1 $\mu\text{g}/\text{mL}$).

Measurement of IL-10 Levels

As shown in Table 5 or figure 5 the addition of ESAT-6 into the blood samples in the positive control group increased IL-10 levels significantly as compared to the negative control ($p < 0.05$). MLE at doses of 1, 2.5, and 10 $\mu\text{g}/\text{mL}$ showed lower levels of IL-10 as compared to the positive control, and the dose of 25 $\mu\text{g}/\text{mL}$ had the IL-10 level higher than that of the positive control.

Table 1: Phagocytic Activity of Macrophages

Sample	MLE Dose ($\mu\text{g/mL}$)	Percentage of Macrophage Phagocytic Activity (%)
NC	-	20.63 \pm 0.26*
PC	-	10.30 \pm 1.24*
P1	1	18.80 \pm 0.91*
P2	2.5	17.68 \pm 0.97*
P3	10	15.53 \pm 0.40*
P4	25	12.77 \pm 0.75*

Note: Data are presented in mean percentage (%) \pm SD. *Significant difference ($p < 0.05$); Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1-P4: PBMC culture + ESAT-6 + MLE

Table 2: Intracellular ROS Level

Sample	MLE Dose ($\mu\text{g/mL}$)	Percentage of intracellular ROS level (%)
NC	-	3.34 \pm 0.29*
PC	-	2.16 \pm 0.79*
P1	1	5.06 \pm 0.45*
P2	2.5	4.32 \pm 0.37*
P3	10	3.09 \pm 0.05*
P4	25	1.65 \pm 0.41*

Note: Data are presented in mean percentage (%) \pm SD. *Significant difference ($p < 0.05$); Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1-P4: PBMC culture + ESAT-6 + MLE

Table 3: NO levels

Sample	MLE Dose ($\mu\text{g/mL}$)	NO levels ($\mu\text{mol/mL}$)
NC	-	17.13 \pm 0.96*
PC	-	12.16 \pm 0.59*
P1	1	15.47 \pm 0.72*
P2	2.5	14.40 \pm 0.20*
P3	10	14.08 \pm 0.66*
P4	25	14.01 \pm 0.60*

Note: Data are presented in mean concentration $\mu\text{mol/mL}$ \pm SD. *Significant difference ($p < 0.05$); Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1-P4: PBMC culture + ESAT-6 + MLE

DISCUSSION

The *M. Olifera* leaves extract (MLE) was evaluated for its immunomodulatory activity in terms of macrophage phagocytosis activity, ROS, and NO levels, and the ratio of IFN- γ and IL-10 in TB patients induced by ESAT-6 antigen in vitro. In the macrophage phagocytosis activity test, the negative control showed high phagocytosis activity of macrophages. This might be due to the optimal phagocytosis ability of macrophages cells (innate immunity) because it was derived from the peripheral blood, which was not directly infected by MTB. The phagocytosis activity of macrophages includes nonspecific cellular immunity. It is stimulated by various stimuli such as microbes and their products, antigen-antibody complexes, inflammation, sensitized T lymphocytes, cytokines, and trauma.¹⁹ ESAT-6 as a pathogenicity factor for MTB was proved to be able to decrease the phagocytic activity of macrophages as shown in the positive control

group. ESAT-6 has an important role in phagosome rupture and MTB cytosolic translocation, which causes impaired phagocytosis activity. ESAT-6 is secreted by MTB through Esx-1/Esx-A (type VII secretion system), which can inhibit phagosome maturation and damage the phagosome membrane so that these bacteria escape the elimination process by phagolysosomes and can cause macrophages cell death.^{3,9}

MLE at all doses used increased the macrophage phagocytic activity significantly when compared to the positive control ($p < 0.05$), but the increase in the activity did not correspond to the increasing dose, on the contrary, decreasing the dose caused an increase in the activity. This indicated that at low doses, MLE had an immunostimulating activity, but at high doses, it showed an opposite activity of immunosuppressive activity. The immunostimulating activity of MLE at low doses might be due to triggering the activity of Mitogen-Activated Protein Kinase (MAPK), which

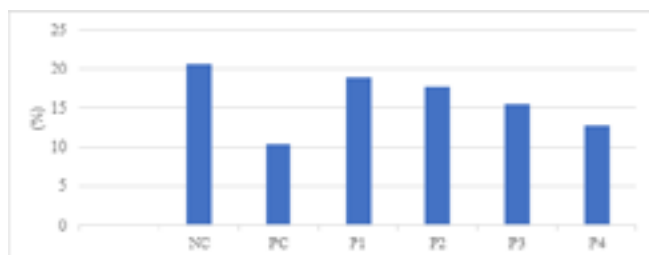


Figure 1: Means percentages level of macrophages phagocytosis activity in each group. Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1: PBMC culture + ESAT-6 + MLE 1 µg/mL; P2: PBMC culture + ESAT-6 + MLE 2.5 µg/mL; P3: PBMC culture + ESAT-6 + MLE 10 µg/mL; P4: PBMC culture + ESAT-6 + MLE 25 µg/mL

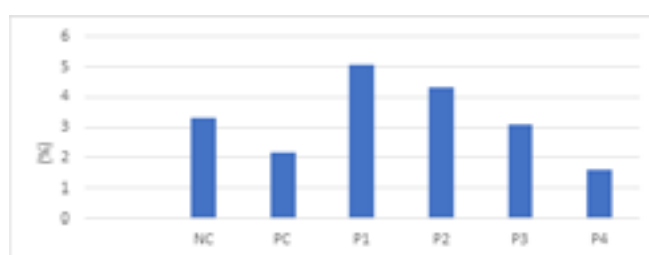


Figure 2: Mean Percentage level of Intracellular ROS in Each Groups. Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1: PBMC culture + ESAT-6 + MLE 1 µg/mL; P2: PBMC culture + ESAT-6 + MLE 2.5 µg/mL; P3: PBMC culture + ESAT-6 + MLE 10 µg/mL; P4: PBMC culture + ESAT-6 + MLE 25 µg/mL

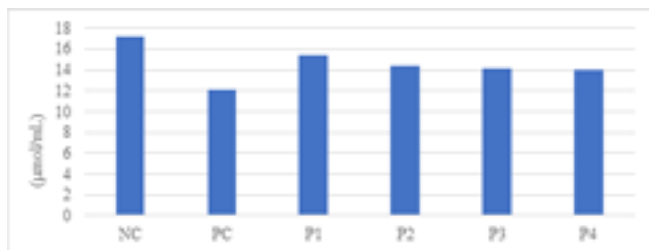


Figure 3: Mean Concentration level of NO in Each Groups. Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1: PBMC culture + ESAT-6 + MLE 1 µg/mL; P2: PBMC culture + ESAT-6 + MLE 2.5 µg/mL; P3: PBMC culture + ESAT-6 + MLE 10 µg/mL; P4: PBMC culture + ESAT-6 + MLE 25 µg/mL

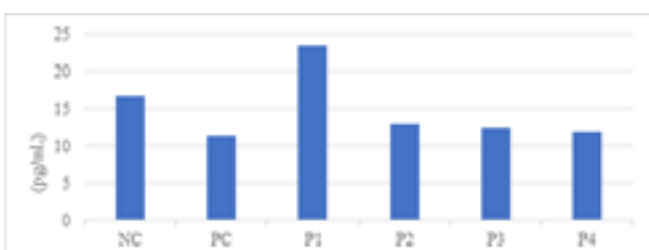


Figure 4: Mean Concentration Level of IFN-γ in Each Groups. Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1: PBMC culture + ESAT-6 + MLE 1 µg/mL; P2: PBMC culture + ESAT-6 + MLE 2.5 µg/mL; P3: PBMC culture + ESAT-6 + MLE 10 µg/mL; P4: PBMC culture + ESAT-6 + MLE 25 µg/mL

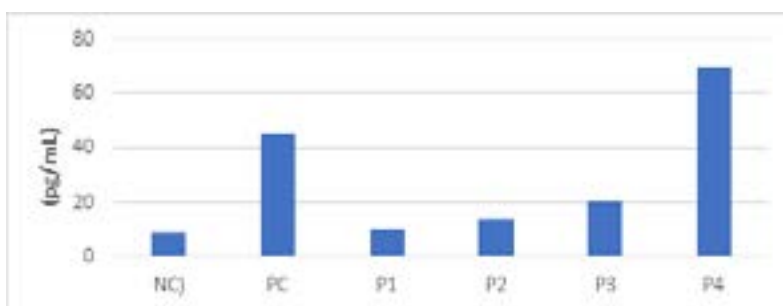


Figure 5: Mean Concentration Level of IL-10 in Each Groups. Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1: PBMC culture + ESAT-6 + MLE 1 µg/mL; P2: PBMC culture + ESAT-6 + MLE 2.5 µg/mL; P3: PBMC culture + ESAT-6 + MLE 10 µg/mL; P4: PBMC culture + ESAT-6 + MLE 25 µg/mL

has a role in stimulating macrophages and the excretion of several proinflammatory cytokines such as TNF- α , IL-12, IFN- γ , and iNOS.^{10,20} The contents of phytochemicals, vitamins, and minerals in MLE might have a role in the immunostimulating or immunosuppressive activity of MLE.

In the measurement of ROS and NO levels, the negative control group showed high levels of ROS and NO, because the PMA induction on PBMC cells, besides stimulating monocyte differentiation into macrophages, also stimulated macrophages to produce ROS and NO. In the positive control group, the intracellular ROS and NO levels decreased

significantly as compared to the negative control, which might be caused by the inhibitory effect of ESAT-6 on the production of intracellular ROS/NO levels. Another researcher found that cofilin 1 as an actin-depolymerizing protein or actin filament, which plays an important role in phagocytosis, phagosome acidification, and phagolysosomal fusion in macrophages cells, decreases due to the MTB-produced ESAT-6, and the decreased cofilin ONE level can reduce intracellular ROS levels in macrophages.²¹ The research conducted by other researchers in Th1 cells and human macrophages induced by ESAT-6 or the combination of

Table 4: IFN- γ levels

Sample	MLE Dose ($\mu\text{g/mL}$)	IFN- γ levels (pg/mL)
NC	-	16,65 \pm 0,13*
PC	-	11,28 \pm 0,11*
P1	1	23,43 \pm 0,80*
P2	2.5	12,98 \pm 0,36*
P3	10	12,35 \pm 0,23*
P4	25	11,76 \pm 0,39*

Note: Data are presented in mean concentration pg/mL \pm SD. *Significant difference ($p < 0.05$); Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1-P4: PBMC culture + ESAT-6 + MLE

Table 5: IL-10 levels

Sample	MLE Dose ($\mu\text{g/mL}$)	IL-10 levels (pg/mL)
NC	-	9.06 \pm 1.50*
PC	-	45.28 \pm 1.17*
P1	1	10.72 \pm 1.64*
P2	2.5	13.94 \pm 2.14*
P3	10	20.39 \pm 0.51*
P4	25	69.72 \pm 1.02*

Note: Data are presented in mean concentration pg/mL \pm SD. *Significant difference ($p < 0.05$); Negative control (NC): PBMC culture without ESAT-6; Positive control (PC): PBMC culture with ESAT-6; P1-P4: PBMC culture + ESAT-6 + MLE

ESAT-6/CFP-10, showed a decrease in intracellular NO and ROS levels.¹⁰

In the intervention groups, MLE at low doses increased the intracellular ROS and NO levels significantly when compared to the positive control ($p < 0.05$), but as the dose increased, the ROS and NO levels decreased. The lowest dose of MLE (1 $\mu\text{g/mL}$) gave the highest intracellular ROS and NO levels. The decreasing effect of MLE on intracellular ROS and NO levels began to be seen at a dose of 2.5 $\mu\text{g/mL}$. These results were the same as the macrophages phagocytosis activity test, where a decrease occurred starting at a dose of 2.5 $\mu\text{g/mL}$.

The immunostimulating effect of MLE at low doses on the increase of intracellular ROS and NO levels was related to the increasing phagocytic activity of macrophages. As stated above that the low doses of MLE increased the phagocytic activity of macrophages. This might be due to the triggering effect of the low doses of MLE on the MAPK activity and nuclear factor-kB (NF-kB) transcription activity, which resulted in increasing the production of intracellular ROS and NO levels.¹⁰

In the measurement of IFN- γ levels, the negative control group had high levels of IFN- γ levels. This might be due to the induction of PMA on the PBMC cells, and then the macrophages and T cells were stimulated to produce IFN- γ . In the positive control group, there was a significant decrease in the IFN- γ levels as compared to the negative control ($p < 0.05$). ESAT-6 can induce IFN- γ but can inhibit IFN- γ production. Another researcher found that ESAT-6 can decrease IFN- transcription and reduce expression of the transcription factor ATF-2 (Activating Transcription Factor-2)

gene and c-Jun gene, which normally binds to the proximal IFN- γ promoter that stimulates mRNA expression in human PBMC cells. ESAT-6 can inhibit the production of IL-17 and TNF- α , as well as increase the production of IL-2. ESAT-6 can directly inhibit T cell response to Ag MTB by interfering with the T cell Receptor (TCR) signaling pathway in ZAP-70. Higher concentrations of ESAT-6 can directly inhibit the production of IFN- γ in response to T cells to MTB infection or can inhibit TCR activation by reducing T cell activation without affecting TCR signaling.²²

The administration of MLE in the intervention groups increased the IFN- γ levels, but the highest and most significant increase was shown by the lowest dose of MLE (1 $\mu\text{g/mL}$). These data corresponded to those of the measurement of macrophages' phagocytosis activity and intracellular ROS and NO levels with the lowest MLE dose of 1 $\mu\text{g/mL}$ previously induced by ESAT-6. The immunostimulating activity of low doses of MLE could increase the intracellular IFN- γ , similar to the argument for increased intracellular ROS and NO levels. The immunosuppressive effect of MLE on the level of IFN- γ began at a dose of 2,5 $\mu\text{g/mL}$. These results were the same with the macrophages phagocytosis activity and the intracellular ROS and NO levels, where a decrease occurred starting at a dose of 2,5 $\mu\text{g/mL}$.

In the measurement of IL-10, ESAT-6 in the PC group showed an increase in the IL-10 levels. Various studies have been carried out which prove that IL-10 levels have increased in patients with active pulmonary TB. Some research found that there was an increase in IL-10 levels in PBMC cells of patients with active pulmonary TB after ESAT-6/CFP-10

induction.^{7,8,23,24} The increase in IL-10 after being induced by ESAT-6 is predicted to be due to a shift from Th1 to Th2 due to decreased CD4 + T cell proliferation,^{7,23} and Regulatory T cells (Tregs), which act as suppressors T cells are involved in increasing IL-10 production.^{7,8} IL-10 is produced primarily by Th2 cells, regulatory T cells (Suppressor T cells/Tregs), macrophages, Th17 cells, B cells, neutrophils, and some dendritic subsets.¹⁹

In the intervention groups, MLE at a dose of 1 µg/mL showed the lowest level of IL-10, and the of 2 µg/mL began to increase the IL-10 level. The dose of 25 µg/mL even gave the IL-10 level higher than that of the positive control. The low dose of MLE gave an immunosuppressive effect on the IL-10 level, but the high dose showed an immunostimulating effect.

The results of this study indicated that virulence and pathogenicity of the ESAT-6 antigen caused a decrease in the macrophage's phagocytosis activity and the intracellular macrophages ROS, NO, and IFN-γ levels, but induced an increase in the IL-10 levels in PBMC cells with active pulmonary TB in vitro. There were significant differences in those parameters measured after ESAT-6 induction in PBMC cultures of adult active pulmonary TB patients. The *M. Olifera* leaves extract (MLE) at a low dose of 1 µg/mL had an immunostimulating effect which increased the macrophage's phagocytosis activity and the macrophages intracellular levels of ROS, NO, and IFN-γ, and immunosuppression of IL-10 levels in PBMC cells. The extract had an immunosuppressive, anti-inflammatory effect, and stimulates CD4+ T cells to differentiate into Th2 cells, starting at a dose of ≥2,5 µg/mL to decrease the macrophages phagocytosis activity and the macrophages intracellular levels of ROS, NO, and IFN-γ, and immunostimulating the levels of IL-10 in PBMC cells of active pulmonary TB patients in vitro.

The limitation of this study is related to the in vitro research, which used PBMC cells of patients with active pulmonary TB outside the patient's body, which may have a different effect from the real situation. In this study, we did not use MTB bacteria directly but only used ESAT-6 as a model for the virulence and pathogenicity of MTB in PBMC cells of active pulmonary TB patients, so it might give different results if the study was carried out directly either in vivo or directly on patients with active pulmonary TB. The use of MLE is only limited to assessing the effect of immunomodulators without paying attention to its composition and content, so it cannot assess in detail the ingredients and compositions that play a role.

CONCLUSION

This study concluded that MLE had a dose-dependent dual opposite immunomodulatory activity in terms of macrophages' phagocytosis activity and the macrophage's intracellular ROS, NO, and IFN-γ levels. At low doses, MLE had immunostimulating activity, but at higher doses, it induced immunosuppressive activity. These findings revealed that MLE has significant potential to be developed and used as a nutritional supplement for TB patients. For this purpose, the low doses showing immunostimulating activity are

recommended for use in clinical applications to the TB patients. However, further studies on MLE are needed to verify its efficacy, such as directly evaluating antibacterial activity on *Mycobacterium tuberculosis*, pre-clinical examination on animals, and clinical trial. In addition, the search for the active compound responsible for the activity is important to be conducted.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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