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American Journal of Microbiological Research IMMUNOMODULATOR POTENTIAL OF MIANA CLAVE (*Coleus scutellarioides* (L) Benth) IN PRVENTION OF TUBURCULOSIS INFECTION Sesilia Rante Pakadang¹, Chatarina Umbul Wahjuni², Hari Basuki Notobroto², Dwi Winarni³, Ressay Dwiyanti⁴, 6, Yadi Yasir^{5,6}, Muhammad Sabir^{4,6}, Mochammad Hatta^{6*} Health Technology Department, Ministry of Health, Makassar, Indonesia Faculty of Public Health, Airlangga University, Surabaya, Indonesia Faculty of Life Sciences and Technology, Airlangga University, Surabaya, Indonesia Department of Microbiology, Faculty of Medicine, Tadulako University, Palu, Indonesia Department of Microbiology and Immunology, Faculty of Medicine, Mulawarman University, Samarinda, Indonesia Molecular Biology and Immunology Laboratory for Infectious Diseases, Microbiology Department, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. Correspondence address: Prof. Mochammad Hatta, MD, PhD, Clin. Microbiologist (hattaram@indosat.net.id) ABSTRACT Aim.

The aim of this study to investigate the immunomodulator effects of miana leaf (*Coleus scutellarioides* (L) Benth) on prevention of Wistar animal model infected by *Mycobacterium tuberculosis* (M.tbc). Method. Samples of white male Wistar rats were divided into 4 groups, the samples were treated with miana leaf extract (EDM) and then infected by intra tracheal M.tbc H37Rv strain and subsequently given a placebo, EDM and GAB (combined Rifampicin and EDM) and healthy control animals were treated with EDM.

In this study we measure the level of Immunomodulatory parameter; the number of T lymphocytes and CD4 T-cells measured by flowcytometry method, the levels of IFN- γ and TNF- α were measured by ELISA and the number of Mtb colonies derived from the rat lung in LJ media. Result. Results showed the increasing of T-lymphocytes, CD4 + T cells, the levels of IFN- γ , TNF- α and decreasing in the number of Mtb colonies after EDM

treatment.

Conclusion, EDM potentially as an immunomodulator with a mechanism to increasing the number of T-lymphocytes, the number of CD4 T-cells, the levels of IFN- γ , TNF- α levels and decrease the number of colonies M.tbc. Positive relationships between variables showed T-lymphocyte proliferation increase the CD4 T-cells then increase IFN- γ and further decreased the M.tbc colony.

Key words: Immunomodulator, Miana leaf (*Coleus scutellarioides* (L) Benth), *Mycobacterium tuberculosis*, CD4, IFN- γ , TNF- α

INTRODUCTION Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360 000 of whom were HIV-positive.

The majority of cases worldwide in 2012 were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. Indonesia is the fifth countries with the largest number of incident cases in 2012 after India, China, South Africa, with incident cases 0.4 million–0.5 million. The detection of new cases of pulmonary TB disease in Indonesia in 2013 was 196 310 cases. In Indonesia, South Sulawesi province becomes seventh ranks with 10.970 new cases of pulmonary tuberculosis patients [1, 2].

TB is slowly declining each year and it is estimated that 37 million lives were saved between 2000 and 2013 through effective diagnosis and treatment. However, given that most deaths from TB are preventable, the death toll from the disease is still unacceptably high and efforts to combat it must be accelerated if 2015 global targets, set within the context of the Millennium Development Goals (MDGs), are to be met [3].

Tuberculosis can infect anyone who lives in the patient environment. People who live and interact with the patient can be infected with *Mycobacterium tuberculosis* (Mtb) by patients droplets inhaled through the air. A person with a healthy condition without immunity impairment will be spared from TB, although it has been exposed.

Activation of macrophages serves to increase phagocytosis, increase the destruction of microorganisms, increase chemotaxis, act as antigen presenting cells, increases the secretion of enzymes and increase the important biological substance. Macrophages are important effector cells involved in phagocytosis, microbial killing and antitumour activity.

Macrophages also display accessory cell function, in that they can present antigen to

foster the development of T lymphocyte-mediated immunity. These Properties may be influenced by cytokines such as IFN- γ and TNF- α released from macrophages, lymphocytes and other tissue cells [4-6]. Macrophage-Myco**ba**cterium interactions and the role of macrophage in host response can be summarized under the following headings: surface binding of M.

tuberculosis to macrophages; phagosome-lysosome fusion; mycobacterial growth inhibition and killing; recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity. The specific acquired immune response mainly responsible for protective Th1 cytokines and through CD8 cells bringing about cytotoxicity.

The complexity of the host-pathogen interaction and underlines the importance of identifying **the mechanisms involved in** protection [7]. It is important to improve immunity status of patients during tuberculosis chemotherapy. **Immunostimulant supplementation may represent a novel approach for fast recovery in tuberculosis patients.**

Immunomodulatory drugs can be chemical or natural products such as herbal remedies derived from plants. The tendency back to nature today, causing the utilization of herbal medicine became very popular in the community. Medicinal plants now used as a primary or complementary therapy to boost immunity or maintain health and fitness.

One of the medicinal plants used in Toraja ethnic communities **(in the province of South Sulawesi, Indonesia) is miana (Coleus** scutellarioides, (L) Benth). Preliminary study in 2013 on practitioners of traditional healers in Tana Toraja Toraja concluded that tribal communities have been using the leaf of miana for TB treatment.

The survey found that 74% of TB patients using traditional medicine as complementary so that miana leaves as become indigenous in the treatment of TB in the Toraja. Although it has been used empirically by the Torajanese for TB treatment but there is no scientific demonstration, then the miana leaf potential to be researched and developed considering tuberculosis cases in Indonesia is still high.

MATERIALS AND METHODS Ethics statement All animals were maintained in accordance with protocols approved by the institutional animal ethical committee of Animal Care and Use Committee of Fakultas Kedokteran Hewan Universitas Airlangga dengan nomor ethical clearance: 364-KE Bacterial strains We using Mycobacterium tuberculosis H37Rv strain obtained from Tuberculosis Laboratory, Institute of Tropical Disease, Airlangga University. 50 μ L of 5×10^8 cells/ml of bacteria was used [8-10].

Miana leaves preparation Processing miana leaves into dry simplicia then extracted by maceration using ethanol 70%, to obtain a pure viscous extract containing chemical components. Further extracts standardized according to the standard of non-specific and specific parameters that it becomes standardized extracts test material (EDM) [11-13].

EDM dose used was 510mg/kg rat suspended with Sodium CMC 1% w/v. Rats Wistar strain white rats are used as test animals were obtained from animal unit of the Faculty of Medicine, University of Airlangga. Animal testing is a male aged 2-3 months weighing 150-200 grams. During the experiment the animals were given feed and water.

Rats were divided 4 groups each of 10 animals. K1 is a group of healthy control animals given EDM for 51 days; K2, K3 and K4 are groups of animals given preventive EDM 21 days later infected with Mtb H37Rv strain (day 22) in intratracheal, day to 22-51 followed by placebo treatment Na CMC (K2); EDM (K3) and the GAB (combined EDM and Rifampin) as K4.

We infected with 50µl of 5x10⁸ unit/ml of Mtb intratracheal in anaesthetized wistar with ketamin (100mg/ml); diazepam (5 mg/ml) intramuskular [8, 10, 14-17]. Immunity parameter Immune parameters tested in this study including the number of T lymphocytes and CD4 T cells from blood samples rats, using flowcytometri methods; IFN γ and TNF α levels from rats blood samples, using ELISA method and the number of Mtb colonies from rats lung samples in Lowenstein Jensens medium.

RESULTS Number cells of T lymphocyte and CD4 T cells among several series of intervention animal. The series 1 as a control group of healthy test animals were given EDM was shown that the mean of number of T lymphocyte and CD4 T cells is 54.67 and 22.16 while series 2 as a control group of sick animals that were given preventive EDM and placebo was shown that the mean of number of T lymphocyte and CD4 T cell is 83.63 and 42.05.

Furthermore, the series 3 as groups of test animals were given preventive EDM pain was shown that the mean of number of T lymphocyte and CD4 T cell is 79.96 and 38.54 while the series 4 as groups of test animals were given preventive EDM pain and GAB was shown that the mean of number of T lymphocyte and CD4 T cell is 58.98 and 28.25. There significantly different among all series group (Figure 1) _ Figure 1.

Mean of number cells of T lymphocyte and CD4 T cells among several series of intervention animal. abcd The same superscript showed no difference between groups

of each variable Series 1: a control group of healthy test animals were given EDM Series 2: a control group of sick animals that were given preventive EDM and placebo Series 3: groups of test animals were given preventive EDM pain and Series 4: groups of test animals were given preventive EDM pain and GAB Level of IFN gamma and TNF alpha level among several series of intervention animal.

The series 1 as a control group of healthy test animals were given EDM was shown that the mean of level of IFN gamma and TNF alpha is 0.72 and 1.22 while series 2 as a control group of sick animals that were given preventive EDM and placebo was shown that the mean of level of IFN gamma and TNF alpha is 6.37 and 140.77. Furthermore, the series 3 as groups of test animals were given preventive EDM pain was shown that the mean of level of IFN gamma and TNF alpha is 2.30 and 73.25 while the series 4 as groups of test animals were given preventive EDM pain and GAB was shown that the the mean of level of IFN gamma and TNF alpha is 0.77 and 9.55.

There significantly different among all series group (Figure 2) __ Figure 2. Mean of IFN gamma and TNF alpha level among several series of intervention animal. abcd The same superscript showed no difference between groups of each variable Series 1: a control group of healthy test animals were given EDM Series 2: a control group of sick animals that were given preventive EDM and placebo Series 3: groups of test animals were given preventive EDM pain and Series 4: groups of test animals were given preventive EDM pain and GAB M.tbc colony among several series of intervention animal.

The series 1 as a control group of healthy test animals were given EDM was shown that the mean of M. tbc colony is 0 while series 2 as a control group of sick animals that were given preventive EDM and placebo was shown that the mean mean of M. tbc colony is 321.88 Furthermore, the series 3 as groups of test animals were given preventive EDM pain was shown that the mean of mean of M.

tbc colony is 129 while the series 4 as groups of test animals were given preventive EDM pain and GAB was shown that the the mean of mean of M. tbc colony is 0. There significantly different among all series group (Figure 3) __ Figure 3. Mean of M.tbc colony among several series of intervention animal. abcd The same superscript showed no difference between groups of each variable Series 1: a control group of healthy test animals were given EDM Series 2: a control group of sick animals that were given preventive EDM and placebo Series 3: groups of test animals were given preventive EDM pain and Series 4: groups of test animals were given preventive EDM pain and GAB The relationship among variable.

The relationship among all variable was shown that significantly different except the

relationship between number of CD4 T cell and M tbc colony; number T lymphocyte and M. tbc colony and level of TNF alpha and M. tbc colony (Table 1). Table 1. Results Analysis of the Relationship among Variables V. Independent _V. Dependent _Coeffisien (standardized) _P __ T lymphocyte __CD4 T cells _0.3973 _0.0304 __CD4 T cells __M. tbc _-0.0876 _0.6008 __CD4 T cells __TNF-a _0.9003 _<0.001 __CD4 T cells _ _IFN-? _0.6970 _<0.001 __T lymphocyte __M. tbc _0.0142 _0.8439 __TNF-a __M. tbc _0.0710 _0.6403 __IFN-? __M. tbc _0.9557 _<0.001 __Description Related Not related In conclusion revealed that results Kruskal-Wallis test showed that there were differences in the number of T lymphocytes, CD4 T cell counts, levels of IFN?, TNFa levels and the number of colonies M.tbc from rats lung samples.

DISCUSSION The effect of EDM on T lymphocyte proliferation in mice models determined based on differences in the number of T lymphocytes of each sample in the treatment group. It is explained that the EDM effect on T lymphocyte proliferation and function as an immunomodulator that acts to increase immunity (immunostimulant). Medicinal plants are plants or parts of plants can strengthen the function of organs, can get rid of toxins or disease and can build up the immune system [18].

One mechanism herbs as complementary and alternative medicine (CAM) in improving immunity or modulate the immune response to pathogens or regulate of T cells [19]. Miana leaves as one of the medicinal plants have been used empirically, in line with the results of studies showing that the EDM can increase the T lymphocytes. T lymphocyte count varies depending on the level of healing. The differences in T lymphocyte proliferation caused by differences in the treatment.

Results showed that there was no differences between group 1 (Healthy animal with EDM) and group 4 (sick animal with EDM and GAB). This happens because of the provision of preventive EDM has triggered the proliferation of T lymphocytes prior to infection, making initial immunity to face the infection.

Cytokines act as the main line to control the initial infection, maintain T-cell responses in mediating immune host [20] and involves all competent immune cells; CD3 (white blood cells), CD4 (helper T cells) and CD8 + (cytotoxic T cells) [21]. IFN-? cytokines produced by lymphocytes, particularly T cells and natural killer cells (NK) [5] acting on T lymphocytes to promote the differentiation of naive T cells to CD4 and Th1 cells. Th1 cells are involved in the elimination of pathogens in intracellular vesicular compartments [22].

The results showed EDM giving effect on the number of CD4 T cells, based on differences in the number of CD4 T cells in each sample treatment group, making EDM

can function as an immunomodulator to prevent tuberculosis. Another mechanism herbal CAM function in improving immunity or immunity is to modify the level and quality of the immune response of T cells, B cells, and cytokines [19]. Giving treatment, alone or complementary EDM (GAB) effect on the number of CD4 T cell production further improvements also affect the host immune.

Anti-tuberculosis immunity dominated by Th1 CD4 cells. Th1 CD4 cell protection capability is based on its ability to secrete IFN γ and other cytokines and then activates macrophages to function as bacteriostatic at the site of infection [23].

Mycobacterium tuberculosis (Mtb) is mainly located in the vacuole of macrophages, will stimulate MHC class II antigen presentation Mtb on CD4 T cells as a result of the infection. Increased response sick animals against infection factors greatly influenced by the immune system when an infection occurs. In this study, the group of sick animals who received curative GAB shows the number of CD4 T cells is the lowest.

EDM has a preventive effect on increasing immunity prior to infection and increase in CD4 T cells into early host defense when infected Mtb. The main function of CD4 T cells is producing IFN γ and other cytokines to activate macrophages. The importance of EDM as a preventive is to improve host immunity before exposure to the infection occurs.

In line with the research North-Jung (2004) in rats showed that at the beginning of the infection the number of MHC Class II or CD4 decline rapidly making the levels of IFN γ is also reduced. Reducing in CD4 T cells is caused by the rapid reactivation of infection. Apoptosis of infected cells by CD4 T cells also play a role in controlling infection. The results showed EDM affect on the increase in IFN γ .

All the treatment of test animals increased the IFN γ compared to healthy control animals. This shows that the EDM as an immunomodulator can improve immunity (immunostimulant). Herbal plant that serves as a CAM can boost immunity or immunity by the mechanisms altering the balance between the inflammatory and anti-inflammatory cytokines [19]. Immunomodulatory function of medicinal plants is determined by the active component contained in the plant cells.

Miana plant as one of the family Lamiaceae medicinal plant containing among other essential oils, flavonoids, tannins and alkaloids potentially as an immunomodulator and proven to increase the production of cytokines such as IFN γ . Immunomodulator activity is determined by knowing the ability of plant extracts to induce NO (nitric oxide), cytokine production, and phosphorylase mitogen activated protein kinase (MAPK) [24].

The importance of increasing IFN γ in the Mtb infected host because the number of Mtb bacteria in the body can be controlled by the immune mechanisms of the host which is stimulated by cytokines IFN γ . Main function of IFN- γ is to participate in the fight against tuberculosis [25]. IFN- γ from T cells inhibits the intracellular replicating Mtb in macrophages. This shows the IFN- γ is necessary for intracellular bactericidal activity.

IFN- γ is required by humans and rats to control Mycobacterium tuberculosis. CD4 T cells are a significant source of IFN- γ during acute infection in rat and are required to control bacterial growth and survival of the host and enhance the function of CD8 T cells during Mtb infection. When a host is infected Mtb, Th1-type cytokines show protection of essential immunity [26].

IFN γ receptor deficiency causes the rats susceptible to Mtb, as well as humans deficient the receptors to produce IFN γ will suffer even lead to death. At the **end of the study found a group of sick animals given preventive EDM and GAB** after infected, showing the number of IFN γ is the lowest compared to other sick animals. This means that at the beginning of the infection the cytokines IFN γ production is sufficient so as to facilitate the process of immunity.

At the end of the infections found cytokine levels are very low compared to other groups of sick animals. IFN- γ is a key cytokine involved in the immune response to mycobacteria with the main function activates macrophages, which makes it possible to deploy a microbicide [27]. The results showed EDM giving effect to the increase of TNF α .

This shows that the EDM as an immunomodulator can improve immunity and prove EDM serves as a complement in the treatment of tuberculosis. Alternative medicine, called CAM (complementary and alternative medicine) can improve the immune system with various stages affect the formation of antibodies and immune complexes, alter the balance of inflammatory and anti-inflammatory, as well as regulate the response of pathogens [19].

Levels of TNF- α cytokine found to be very low, especially in the group of sick animals given GAB compared to sick animals' control. Results showed that the number of Mycobacterium tuberculosis in the body can be controlled **by the host immune mechanisms**, because in the process of mycobacteria infection control, TNF α has a primordial function.

TNF α synergize with IFN γ stimulates the **production of reactive nitrogen intermediates (RNIS)**, thereby inhibiting the function of macrophages in tuberculosis mediation,

stimulate the migration of immune cells to the site of infection and contributes to the formation of granulomas that control disease progression [27]. Increased IFN- γ , TNF- α , and IL-6 proved to reduce infection caused by Mycobacterium tuberculosis [28], and an increase in cytokines influenced by the increase in titer of bacteria [29].

Elevated levels of TNF- α that is substantially in CD4 + T cells in subjects infected with Mtb is the strongest predictor of diagnosis of active disease compared latent infection [26]. Immunoregulatory function for sick animals that received preventive EDM and GAB positively affect host defenses against latent infection, so that the levels of cytokines at the end of the treatment approach of healthy control animals as TNF α production in accordance with the degree of illness.

This is consistent with the conclusions of TB patients TNF- α production at every level of the disease [30]. The results showed EDM effect on decreasing the number of colonies of Mycobacterium tuberculosis. In this study demonstrate that administration of EDM as rifampicin complement results did not differ from healthy controls and prove EDM serves as a complement in the treatment of tuberculosis.

If the host has been no initial immunity, this allows the tuberculosis bacteria multiply and spread through the lymphatic vessels and blood flow. When a host is infected Mtb then partly tuberculosis bacteria undergo phagocytosis by macrophages in the alveolar but have not been able to kill bacillus, so the bacilli in macrophages generally can survive and multiply.

Tuberculosis bacteria are spread through the lymph vessels reach the regional lymph nodes, whereas that through the blood stream to reach the various organs of the body, and in the organ will be processed and transfer the antigens to the lymphocytes. Mycobacterium tuberculosis almost always be lodged in **the bone marrow, liver,** lymph nodes, but did not always able to proliferate extensively, whereas in the field of tuberculosis bacteria on the lung, kidney, bone and brain more easily breed mainly before immunity is formed [31].

Mycobacterium tuberculosis that infect the host actually be engulfed by macrophages as the natural defenses are then formed granulomas to kill and prevent the spread of this bacteria. The process of granuloma formation and destruction of bacteria involves the immune mechanisms such as the proliferation of T lymphocytes, CD4 **T cells and the production of cytokines** such as TNF- α and IFN γ . Granuloma is a compact aggregate of immune cells.

These cells act as the immune protection (as a defense wall of the tuberculosis bacteria).

An understanding of the mechanisms and effects of granuloma formation can guide the development of therapies to modulate the formation of granulomas [32]. Based on Amos path analysis, found that the increased proliferation of T lymphocytes affect the increase in the number of CD4 T cells, but does not affect to the reduction in the number of Mtb colonies.

Although the EDM significantly affecting the increased proliferation of T lymphocytes, but it turns out the increase in T lymphocytes did not directly affect a decrease in the number of Mtb colonies. The antibacterial properties of the herbs plant derived from the chemical content in cells, but the mechanism of bacterial killing in the host influenced by mechanisms of host immunity. EDM has been shown to increase proliferation of T lymphocytes but did not exhibit significantly in reducing the number of Mtb colonies directly.

CD4 T cells, cytokines IL-12, IFN γ and TNF are essential in Mtb infection control, but a host of factors that determine why some individuals are protected from infection while the other hosts continue to develop the disease. Path analysis also found that the increase in the number of CD4 T cells affect the increased levels of IFN γ and TNF- α levels.

Using EDM as a preventive has served to increase host immunity such as T lymphocytes, CD4 T cells, cytokines IFN γ and TNF- α . Host immune response to Mtb mediated by cellular immunity, in which cytokines and Th1 cells play an important role [27]. This suggests that the mechanism of immunity that occurs in the the host is complex, making EDM effect on the host immune mechanisms increase in T lymphocytes, CD4 T cells, cytokines IFN γ and TNF α is essential to support the healing of tuberculosis.

Further path analysis showed that elevated levels of cytokines IFN γ affect on decreasing the number of Mtb colonies. This means giving EDM as a preventive can serve as an immunomodulator that increases immunity before infected rats. With increased immunity, the body will always be ready to fight the antigen that enters the body including Mycobacterium tuberculosis infectious.

It can be assumed that the preventive administration of EDM as an immunomodulator can prevent a host infected with tuberculosis from the neighborhood. This assumption is in line with the statement that a person with a good immunity conditions, will be spared from tuberculosis, although it has been exposed. One of the mechanisms of the host immunity against tuberculosis is phagocytosis by macrophage cells.

Increased capacity of phagocytosis by phagocytic cells and the activity of phagocytosis

by macrophages, among others influenced by cytokines such as interferon (IFN) and tumor necrosis factor (TNF) [5, 6]. Paths which are positively correlated in the preventive group is the proliferation of T lymphocytes affect on increases the number of CD4 T cells which further increase levels of IFN ? and then decrease the number of Mtb colonies.

CONCLUSION Miana leaves extract (Coleus scutellarioides, (L) Benth.)

increased the number of T lymphocytes, CD4 T cell counts, levels of IFN ?, TNF a levels and decreased in the number of M.tbc colony in infected Wistar lung.

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