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The Mango's Mistletoe Leaves Extract Ameliorates Lupus by Inhibiting the Anti-dsDNA Antibody Production, the Percentages of $CD8^+CD28^-$ and $CD4^+CD28^-$ T Cells

Kusworini Handono[1], Sri Sunarti[2], Mirza Zaka Pratama[3], Saiful Hidayat[4]*, Muhammad Badrus Solikhin[4], Inmas Andi Sermoati[5], Maria Gabriela Yuniati[5]

[1]Departemen of Clinical Pathology, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia; [2]Department of Internal Medicine, Geriatric Division, Brawijaya University, Malang, East Java, Indonesia; [3]Department of Internal Medicine, Rheumatology and Immunology Division, Brawijaya University, Malang, East Java, Indonesia; [4]Magister Program in Biomedical Sciences, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia; [5]Magister Program in Midwife Sciences, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia

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***Correspondence:** Saiful Hidayat, Magister Program in Biomedical Sciences, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia.
E-mail: mimoy_fatah@student.ub.ac.id
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Abstract

BACKGROUND: In SLE patients, repeated antigen stimulations induce a progressive reduction in CD28 expression on the surface of T cells and the chronic inflammation condition. Mango's mistletoe is a parasitic plant that has anti-inflammation, antiproliferation, and immunomodulatory activities.

AIM: This study aimed to investigate the effect of mango's mistletoe leaves extract (MLE) in inhibiting anti-dsDNA antibodies and ameliorating the percentages of $CD8^+CD28^-$ and $CD4^+CD28^-$ T cells in a pristane-induced lupus mice model.

METHODS: Lupus induction was undertaken by an injection of pristane 0.5 ml intraperitoneally in 6–8-week-old female balb/c mice. Mice with lupus signs were grouped randomly into the treatment groups which received MLE at doses of 150, 300, and 600 mg/kgbw/d for 28 days, respectively, and the positive control group without MLE. On day 29, anti-dsDNA antibody levels were analyzed using an ELISA. One of the immunosenescence markers ($CD28^-$ T cells) was investigated using a flow cytometer. ANOVA test was used for statistical analysis.

RESULTS: The mango's mistletoe leaves extract (MLE) significantly decreased the number of anti-dsDNA antibodies ($^*p < 0.05$), the percentages of $CD8^+CD28^-$ T cells ($^*p < 0.05$) and $CD4^+CD28^-$ T cells ($^*p < 0.05$).

CONCLUSION: We resume that the mango's mistletoe leaves can ameliorate lupus by inhibiting anti-dsDNA antibody production and the percentages of $CD8^+CD28^-$ and $CD4^+CD28^-$ T cells.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies directed against diverse nuclear components that affect almost all internal organs and tissues and have a significant impact on patients' survival [1], [2]. Around the world, North America has the highest estimates of SLE incidence and prevalence (23.2/100,000 person/year and 241/100,000 people, respectively [3]. The patients with SLE have a significant burden as a result of the disease's multi-organ involvement and the therapeutic options available [4]. All-cause standard mortality rates in SLE patients increased 2.6 times [3]. The increased risk of mortality is due to infection, renal disease, and cardiovascular disease [3].

Genetic factors, epigenetic changes (post-translation modification of histones and DNA methylation), ultraviolet rays, demethylating drugs (Azacitidine, Decitabine), viral infection, and the higher concentration of hormones such as estrogen and prolactin are the primary etiological factors that cause SLE [5]. SLE is most common in young females of childbearing age (15–45 years), with a female-to-male ratio of 9:1 [6]. These etiological factors operate as a trigger for normal cells to undergo apoptosis, which causes the double-stranded DNA (dsDNA) to be broken, which, in turn, the broken DNA is presented on dendritic cells (DCs) as an antigen [5]. The plasmacytoid DCs (pDCs) stimulate monocytes to become effective antigen-presenting myeloid DC (mDC) by secreting IFN-α [7]. In the secondary lymphoid organs, mDC rapidly catches apoptotic cells and presents autoantigens to $CD4^+$ T cells, which

became activated, proliferated, and underwent clonal expansion [7]. Following that, autoantibodies are generated by B lymphocytes triggered by contacts with autoreactive $CD4^+$ T cells and mDC [7]. In turn, the autoantibodies form immune complexes with neutrophil products and nucleosome components and directly trigger toll-like receptors on pDC, which are stimulated to secrete more IFN-α, thereby spreading the inflammatory response [7]. BAFF also stimulates B cell proliferation and differentiation which, in turn, produces immunoglobulins, and prolongs the survival time of self-reactive B cells [8], [9].

The $CD8^+$ T cells are essential for the detection and elimination of intracellular pathogens [10]. CD28 costimulation in T cell activation is considered to amplify the response to antigen and its absence could have a significant impact on the immunological response [10], [11]. The $CD8^+CD28+$ T cell is a cytotoxic T lymphocyte population [12]. The loss of the CD28 molecule may be caused by a pro-inflammatory environment and repeated antigen stimulation [1]. If there is a repeated stimulation by the same antigen, it causes the progressive loss of CD28, eventually resulting in the formation of $CD28^-$ T cells with shortened telomeres [10], [13]. The highly antigen-experienced or terminally differentiated $CD8^+CD28^-$ T cells own granzyme A/B and perforin play cytotoxic activity, produce proinflammatory cytokines such tumor necrosis factor- and IL-6, and have enhanced CTLA-4 molecule expression [12]. The $CD8^+CD28^-$ T cell population is also very common with autoimmune diseases, chronic infections, and aging [14]. However, these senescence T cells, according to the Kalim *et al*. [15] play minimal roles in SLE disease activity, notably in terms of KLRG1 and $CD57^+$ on both $CD4^+$ and $CD8^+$ T cells. The $CD8^+CD28^-$ ($CD8^+CD57^+$) T cell population is heterogeneous and made up of functionally conflicting (cytotoxic and immunosuppressive) subsets, the overall effect of $CD8^+CD28^-$ ($CD8^+CD57^+$) T cells is determined by the prevalence of a certain subset such as CD134 (OX40), CD137 (4-1BB) and CD278 (ICOS) Il-2 and IL-15, expression of inhibitory NKRs, expression of KLRG1, programmed death 1, expression of phosphoinositide 3-kinase, Bcl-2, and heat-shock protein 27 [13]. Furthermore, the engagement of CD28 on $CD4^+$ T cells increases T cell sensitivity to antigen receptors, increases IL-2, and promotes cell survival by inducing Bcl-XL expression [10]. The $CD4^+CD28^-$ T cells producing IFN-γ are autoreactive and they also express CD161 which aids in tissue invasion [13].

Meanwhile, conventional SLE treatments, such as non-steroidal anti-inflammatory medications, glucocorticoids, hydroxychloroquine, and immunosuppressive agents, are geared at treating symptoms rather than targeting the fundamental etiology of the disease [9]. In addition, they have toxic side effects [16]. Therefore, for the development of new drugs, the use of natural products has received attention as a therapeutic approach for SLE [5]. The alkaloids, flavonoids, terpenoids, and polyphenols found in the mango mistletoe leaf (dendrophthoe pentandra) have drawn interest for their possible health advantages and pharmacological properties such as anti-inflammatory, antioxidant, anticancer, and immunomodulatory properties [17].

The objective of this research is to compare the anti-dsDNA antibody levels, the percentages of $CD8^+CD28^-$ and $CD4^+CD28^-$ T cells between the groups given with the mango' mistletoe leaf extract and the untreated groups (the positive controls). Pristane-induced lupus (PIL) mice model was used in this study to obtain ascitic fluid enriched in anti-DNA autoantibodies and to evaluate the importance of novel therapeutical goals. The disadvantage of PIL is the difficulty to gain all lupus features due to SLE heterogeneity [18], [19].

Materials and Methods

The mango's mistletoe leaves extract

The mango's mistletoe leaf was obtained from the herbal laboratory of Materia Medica, Batu, Indonesia. The drying of 800 grams of the mango's mistletoe leaf at room temperature was carried out for 5 days and then ground into a powder. To create a solid extract, the dried powder was macerated for 72 h in 96% ethanol solvent, filtered, and then evaporated using a rotary evaporator at 45°C.

Animal study groups and lupus induction

Samples were recruited from animal centers from Malang, Indonesia. Criteria for selecting the subjects were as follows: 6–8-week-old female Balb/c mice, white fur, healthy, active movement, weight in the range of 25 and 30 grams, and no pregnant. Mice that died during the experiment were taken out. The treatment of mice was carried out at the animal laboratory, Faculty of Medicine, Brawijaya University. Mice were accustomed to the new climate for 7 days before the beginning of the research. The temperature in the room was kept between 24 and 25°C, with a relative humidity of 50–55%. On a 12 h light/12 h dark cycle, the mice were housed in five cages with free access to food and water. Lupus induction was done by a single injection of pristane 0.5 ml intraperitoneally (Sigma Aldrich, Cat number P2870). Twenty weeks after the injection, the mice were evaluated for SLE clinical and serological features such as alopecia, arthritis, serositis, and the increased anti-dsDNA antibody levels. In addition to the healthy mice group, mice with lupus symptoms were clustered into four groups at random: A positive control group and three therapy groups (P150 mg, P300 mg, and P600 mg). Each group consisted of five mice. The

experiment was approved by Brawijaya University's Ethics Committee (No.160-KEP-UB).

The administration of the mango's mistletoe leaves extract (MLE)

The mango's MLE was dissolved in 0.5 ml aqua and orally given in the intervention groups using a feeding cannula at the dosages of 150 mg, 300 mg, and 600 mg/kgbb/d, respectively, once a day for 28 days. Meanwhile, the negative and positive control groups only received 0.5 ml aqua.

Measurement of anti-dsDNA antibody levels using an enzyme-linked immunosorbent assay (ELISA)

On day 29 of the experiment, mice were sacrificed under ether anesthesia. Their heart blood was collected for further analysis. Mouse anti-dsDNA ELISA kits (MyBioSource, Cat No. MBS269288) were used to assess anti-dsDNA antibody levels in cardiac blood serum according to the manufacturer's protocols. A result was read utilizing the automatic ELISA microwell strip reader (Stat Fax®) and was expressed as IU/Ml.

Senescent T cells measurement using flow cytometry

Initially, splenocyte isolation from balb/c mouse spleen was performed aseptically after the mouse was sacrificed under deep ether anesthesia. Samples were prepared according to the Biolegend protocol. The spleen samples were colored with CD4 anti-mouse PE (Biolegend, Cat number 100408), CD8 anti-mouse PerCP (Biolegend, Cat number 140417), and CD28 anti-mouse FITC (Biolegend, Cat number 122008). The flow cytometry was carried out with BD FACSMelody by the manufacturer's instructions. The percentages of $CD8^{+}CD28^{-}$ and $CD4^{+}CD28^{-}$ T cells were generated using FACSChorus software.

Statistical analysis

Homogeneous and normally distributed data were analyzed utilizing the ANOVA test using the SPSS 25 software followed by the Tukey *post hoc* test. The result was statistically significant if $p < 0.05$.

Results

SLE is characterized by a loss of tolerance of B and T cells to self-antigens, resulting in polysystemic inflammation [20]. The SLICC classification criteria for SLE meet at least four criteria, with at least one clinical criterion and one laboratory criterion OR Lupus nephritis as the only clinical criterion in the presence of ANA or anti-dsDNA antibodies [21], as shown in Table 1 and Figure 1.

Effect of the mango's mistletoe leaves on anti-dsDNA antibody levels

Antibodies against dsDNA are commonly used to diagnose and monitor the progression of SLE patients [2]. As shown in Figure 2, anti-dsDNA antibody

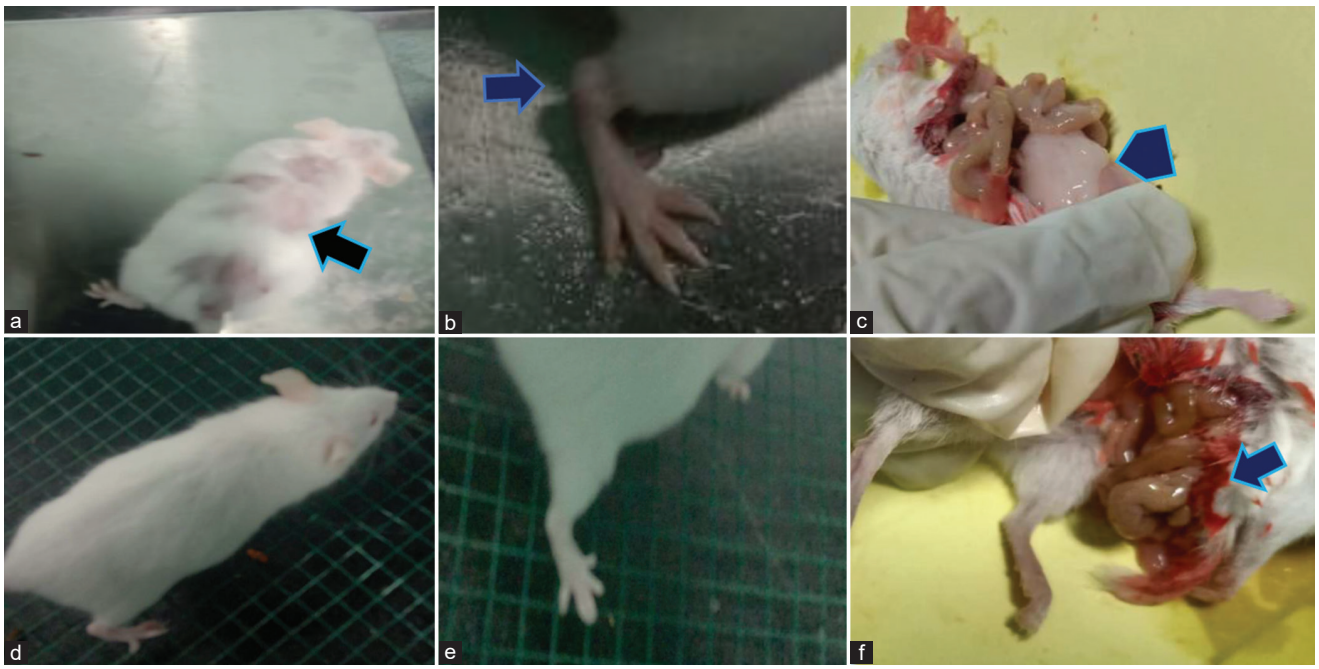

Figure 1: Clinical manifestations of LES mice after pristane injection (0.5 ml of intraperitoneal). SLE mice: (a) Alopecia, (b) arthritis, and (c) serositis; normal mice: (d) Normal hair, (e) normal legs, and (f) no serositis

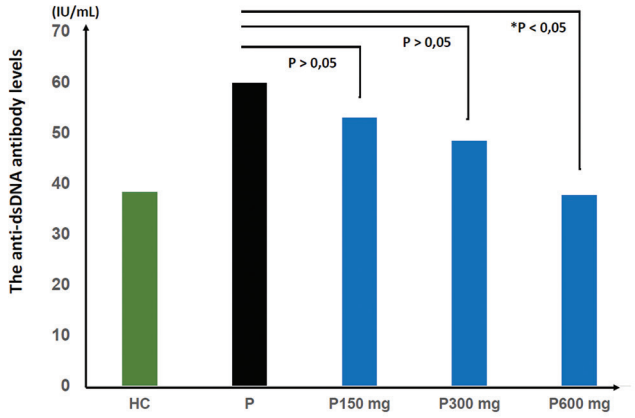

Figure 2: *Effect of the mango's mistletoe leaves extract on anti-dsDNA antibody levels. Anti-dsDNA antibody levels in the group treated with 600 mg of the mango mistletoe leaves extract were significantly lower than the positive control group (*p < 0.05). HC group: Healthy group, P group: Positive control group, Treatment groups: P150 mg, P300 mg, and P600 mg (the administration of mango's mistletoe leaves extract at dosages of 150, 300, and 600 mg/kgbw/d). *p < 0.05, **p < 0.01, and ***p < 0.001. The percentage of CD8$^+$CD28$^-$ T cells*

levels were greater in the positive controls than in the healthy controls. The most interesting aspect of this figure was the anti-dsDNA antibody levels in the group with treated 600 mg of MLE which were considerably lower than in the positive controls (*p < 0.05). This result suggested that the administration of MLE at a dose of 600 mg suppressed the formation of anti-dsDNA antibodies.

Effect of the mango's mistletoe leaves on the percentage of CD8$^+$CD28$^-$ T cells

Expansion of CD8$^+$CD28$^-$ T cells represented terminally differentiated effector lymphocytes [13]. CD8$^+$CD28$^-$ (CD8$^+$CD57$^+$) T cell population is heterogeneous and composed of various functionally competing (cytotoxic and immunosuppressive) subsets thus the overall effect of CD8$^+$CD28$^-$ (CD8$^+$CD57$^+$) T cell depends on the predominance of a particular subset [14]. In this research, positive controls had a higher percentage of CD8$^+$CD28$^-$ T cells than healthy controls (Figure 3). The percentages of CD8$^+$CD28$^-$ T cells in groups given with MLE at dosages of 150 mg and 300 mg were significantly lower compared to the positive controls (**p < 0.01 and *p < 0.05). This outcome exhibited that MLE acted a role as an immunosuppressive agent in the PIL mice.

Effect of the mango's mistletoe leaves on the percentage of CD4$^+$CD28$^-$ T cells

CD4$^+$CD28$^-$ T cells were utilized to predict damage in patients with SLE [1]. In this research, it was discovered that the positive control had a larger percentage of CD4$^+$CD28$^-$ T cells than the healthy controls (Figure 4). As shown in Figure 4, the percentage of CD4$^+$CD28$^-$ T cells in the group treated with 300 mg of MLE was significantly lower when compared to the positive controls. (*p < 0.05). This outcome exhibited that MLE acted a role as an immunosuppressive agent in the PIL mice.

Discussion

In this study, we explore how the mango's MLE inhibits the anti-dsDNA antibody production and ameliorates percentages of CD8$^+$CD28$^-$ and CD4$^+$CD28$^-$ T cells in a PIL mice model. The results of our research revealed that the treatment of lupus with MLE significantly reduced the anti-dsDNA antibody levels, percentages of CD8$^+$CD28$^-$ and CD4$^+$CD28$^-$ T cells.

B cell lymphocytes that activate plasma cell differentiation to produce anti-dsDNA antibodies are significantly inhibited by MLE at a dose of 600 mg. In our study, we presume that the mechanisms are related to the active compounds found in the mango's mistletoe leaf having anti-inflammatory, anti-proliferative, and antioxidant properties [17]. The most of the flavonoid compounds found in the mango's mistletoe leaf interfere with metabolism, proliferation, survival, growth of B cells through the Akt/mTOR and Ras/ERK signaling pathways [22], [23], inhibit BAFF receptors on B cells [8] and obstruct the production of IFN-α, and BAFF by inhibition of pDCs [22], [24], [25]. In addition, a flavonoid also interferes with mDCs to activate T cells and IFN-Y, IL-17, and IL-21 production by T cells [22], [26]. Our findings are consistent with the finding of another study by Guritno *et al*. [27] who discovered that Nigella sativa containing flavonoids and polyphenols could block the production of anti-dsDNA antibodies [28].

The highly antigen-experienced or terminally differentiated CD8$^+$CD28$^-$ T cells are one of the hallmarks of immunosenescence that plays cytotoxic activity and secrete proinflammatory cytokines [12], [29]. The increased number of CD4$^+$CD28$^-$ T cells is correlated with lupus clinical activity and may predict disease damage [1]. Controlling CD28(–) T-cells is critical for successful SLE care and the reaction may be predicted by looking at the intervention that was able to reduce this population [14]. In this study, when compared to the positive controls, the percentage of CD8$^+$CD28$^-$ T-cells was significantly lower in the treatment groups at doses 150 and 300 mg than in the positive controls. We also discovered that the percentage of CD4$^+$CD28– T cells was significantly lower in the group treated with 300 mg of MLE compared to the positive controls.

Coleman *et al.* reported that apoptotic

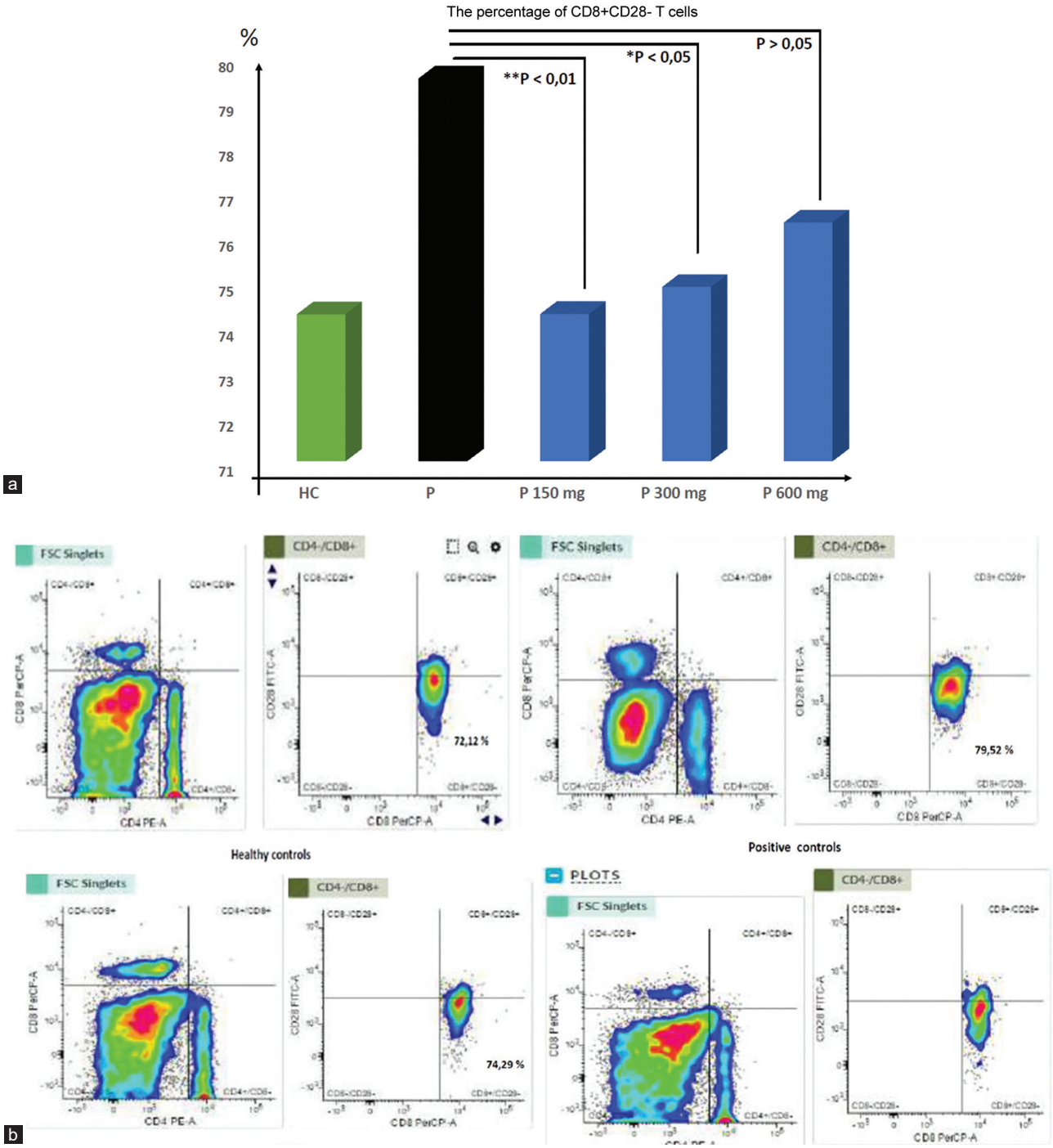

*Figure 3: Effect of the mango's mistletoe leaves extract on the percentage of $CD8^+CD28^-$ T cells in the treatment groups. (a) The percentages of $CD8^+CD28^-$ were lower in groups treated with the mango mistletoe leaves extract at dosages of 150 mg and 300 mg than the positive control group (**p < 0.01, *p < 0.05, and p > 0.05, respectively). (b) Representative of flow cytometric dot plot analysis between groups for the percentage of $CD8^+CD28^-$ measured in the lower right quadrant. HC group: Healthy group, P group: Positive control group, Treatment groups: P150 mg, P300 mg, and P600 mg (the administration of mango's mistletoe leaves extract at dosages of 150, 300, and 600 mg/kgbw/d). *p < 0.05, **p < 0.01, and ***p < 0.001*

resistance of $CD28^-$ T cells depends on a decrease in the pro-apoptotic molecule, Fas, Bim, and Bax, or an increase in the anti-apoptotic molecule Bcl2 [30]. The mango's mistletoe leaf extract (MLE) can reduce the percentage of $CD28^-$ presumably by increasing the BAX/BCL2 ratio and BAK expression [22], [31]. In addition, MLE presumably decreases TNF-α which interferences CD28 expression on T cells [22], inhibits IL-15 and IL-6 that promote the development and maintenance of $CD28^-$ T cells [30], [32], and inhibits P38, ERK1/2, and STAT 3 which play a role in immunosenescence [22], [33], [34]. This result is in line with that of the previous research that quercetin treatment of murine DCs resulted in a decrease in

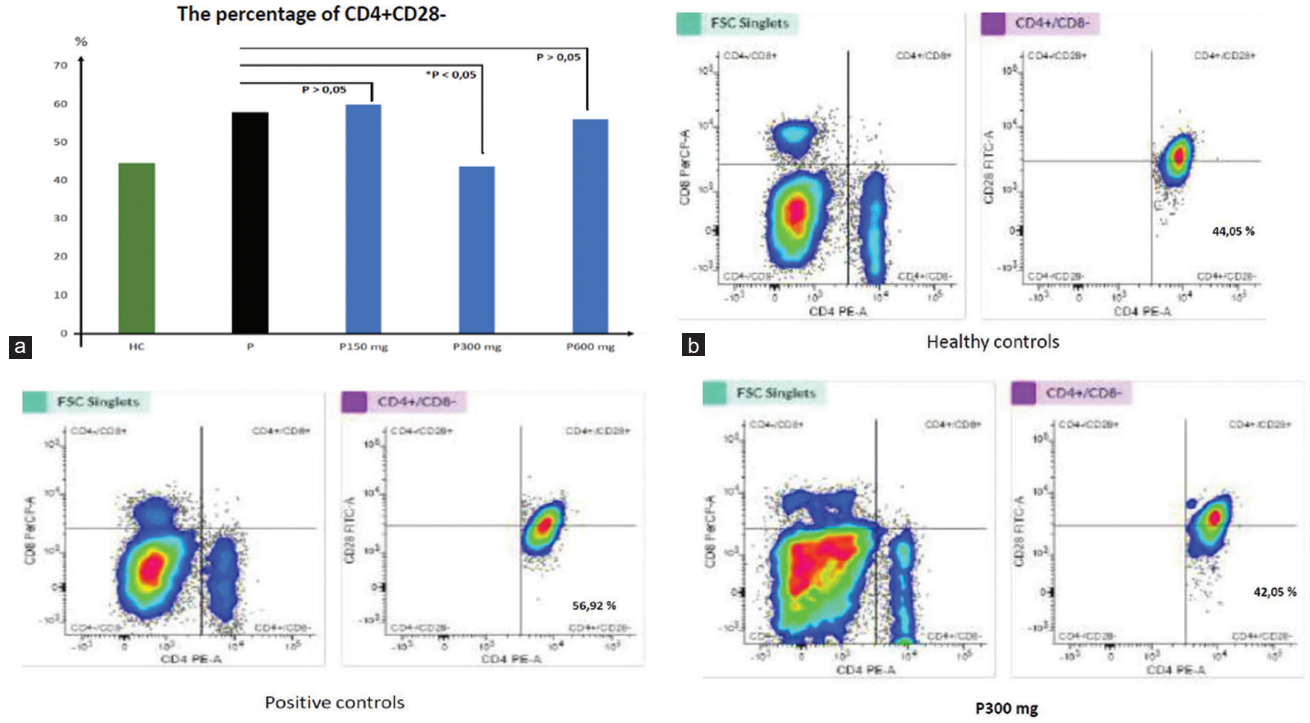

Figure 4: *Effect of the mango's mistletoe leaves extract on the percentage of $CD4^+CD28^-$ T cells in the treatment groups. A. The group administered the mango's mistletoe leaves extract at a dose of 300 mg had a considerably lower percentage of $CD4^+CD28^-$ than the positive control group (*p < 0.05). B. Representative of flow cytometric dot plot analysis among groups for the percentage of $CD4^+CD28^-$ that was measured in the lower right quadrant. HC group: Healthy group, P group: Positive control group, Treatment groups: P150 mg, P300 mg, and P600 mg (the administration of mango's mistletoe leaves extract at dosages of 150, 300, and 600 mg/kgbw/d). *p < 0.05, **p < 0.01, and ***p < 0.001*

Table 1: Characteristics of LES mice at week 24

Group	Healthy controls	Positive controls	150 mg	300 mg	600 mg
Number of mice with alopecia +	0	5	5	5	5
Number of mice with artritis +	0	5	5	5	5
Number of mice with serositis +	0	5	5	5	5
Average weight (gram)	34.72	27.15	32.15	32.94	33.11
The anti-dsDNA antibody levels	38.26 ± 8.66 IU/ml	59.78 ± 18.35 IU/mL	52.86 ± 4.99 IU/mL	48.37 ± 7.59 IU/mL	37.6 ± 5.87 IU/mL

the production of pro-inflammatory cytokines and chemokines, as well as a decrease in the expression level of MHC II and costimulatory molecules, resulting in the reduction in T-cell activation [35]. In conclusion, MLE increases the immunosuppressive effect on $CD28^-$ T cells both $CD8^+$ and $CD4^+$.

Conclusion

This study demonstrates that the percentages of $CD4+CD28^-$, $CD8^+CD28^-$ T cells and the anti-dsDNA antibody levels in the group treated with MLE are significantly lower compared to the positive controls.

The limitations of this study obtained the opposite effect which the administration of MLE at a dose of 600 mg significantly decreased anti-dsDNA antibody levels but did not significantly reduce the percentages of $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells. In the future, greater efforts will be required to examine IFN-α and the safety of MLE in the treatment of SLE as well as this study should be carried out again for a longer time.

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