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EVALUATION OF T LYMPHOCYTE AND REGULATORY T CELL EXPRESSION IN WISTAR RAT POST 90 DAYS OF CHERAL® ADMINISTRATION ORALLY

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ABSTRACT

This study aimed to assess the impact of administering Cheral® (*Curcuma longa* and *Phyllanthus niruri*) on the expression of T lymphocytes and regulatory T cells. The research was conducted in vivo using 24 healthy female Wistar rats, which were categorized into four treatment groups: normal (control), dose 1 (156.25 mg/Kg BW), dose 2 (312.5 mg/Kg BW), and dose 3 (468.75 mg/Kg BW). The rats were orally administered Cheral® for a period of 90 days. T lymphocytes and regulatory T cell profiles were analyzed using flow cytometry. The data obtained were subsequently subjected to analysis using SPSS *software* with One-way ANOVA and Post hoc tests (p-value <0.05). The profiles of T lymphocytes and regulatory T cells following Cheral® administration exhibited diverse outcomes when compared to the normal group. Specifically, dose 2 led to a significant increase in CD4, while dose 1 resulted in a significant increase in CD8. However, the profile of regulatory T cells after Cheral® administration did not exhibit any significant differences when compared to the control group. In conclusion, this research indicates that the compounds present in Cheral® have the potential to modulate the immune system through their effects on T lymphocytes and regulatory T cells.

Keywords: Cheral®, Immune system, T lymphocyte, Regulatory T cell

INTRODUCTION

T lymphocytes are a very important component of the body's defense system. T lymphocytes are needed to recognize and fight infections, as well as regulate the body's immune response. There are various types of T lymphocytes, including T helper cells (CD4) that assist in immune regulation **(Chatzileontiadou *et al.*, 2020)** and cytotoxic T cells (CD8) that destroy infected or mutated cells **(Rout *et al.*, 2024)**. Immature T lymphocytes in the thymus simultaneously carry CD4 and CD8 molecules, so at this stage, they are called double positive ($CD4^+CD8^+$). Double-positive T cells, or immature T cells, will differentiate into single-positive T cells, either CD4 or CD8, depending on their interaction with the major histocompatibility complex (MHC) **(Hagen *et al.*, 2023)**. T cells mature and become fully functional in the thymus, and once mature, they are capable of rapid cell division **(Tabilas *et al.*, 2023)**. Naive T cells, which have not been exposed to antigens, express the CD62L molecule, and activated T cells lose CD62L expression **(Merkenschlager *et al.*, 2023)**.
T cells can express the T Cell Receptor (TCR) and may express the CD8 glycoprotein on their surface, referred to as CD8 T cells (cytotoxic) or CD4 glycoprotein called CD4 T cells or T helper (Th) cells **(Sun *et al.*, 2021)**. Th cells can differentiate into several subtypes, including Th1, Th2, Th9, Th17, Th22, regulatory T cells (Treg), and follicular helper T cells (Tfh). Each of these cells is characterized by different cytokine profiles **(Tuzlak *et al.*, 2021)**. T cells' cytotoxic function is to kill pathogens and virus-infected cells. Th cells assist in activating B cells to produce antibodies, while Treg cells can suppress the immune system's activity in other lymphocytes to prevent excessive immune reactions **(Kervevan & Chakrabarti, 2021)**. As cells are involved in immune system homeostasis, Treg cells also express various biomarkers that play a role in the inflammatory response. These biomarkers specifically inhibit the appropriate cells in the immunosuppressive mechanism, such as TGF-β, IL-10, and IL-35 **(Xia *et al.*, 2021)**.
Curcumin, a compound found in *Curcuma longa* (turmeric), has the potential to act as an anti-inflammatory, antioxidant, anticancer agent, and immunomodulator **(Razavi *et al.*, 2021)**. Curcumin can enhance T-cell proliferation as an anti-inflammatory agent, curcumin can act as a cyclooxygenase inhibitor, reducing prostaglandins and downregulating proinflammatory cytokines **(Makuch *et al.*, 2021)**. *Phyllanthus niruri* contains the bioactive compound such as Phyllanthin, which has been shown to enhance the number of B and T lymphocytes through the ERK MAPK pathway, which is involved in growth, differentiation, and proliferation. **(Hermansyah *et al.*, 2023)**. It can also increase the activity of macrophages and lymphocytes. Flavonoids are the main active compounds in Phyllanthin. These flavonoid compounds have the potential to act as antiviral, anti-inflammatory, anticancer, antioxidant, and immunomodulatory agents **(Marhaeny *et al.*, 2021)**. Phyllanthin can stimulate the innate immune response by activating natural killer (NK) cells and reducing the maturation and differentiation of dendritic cells. On the other hand, flavonoids in Phyllanthin also regulate the adaptive immune response by facilitating the proliferation of regulatory T cells **(Reza *et al.*, 2023)**.
Herbal medicines differ from chemical medications, as they do not produce immediate effects. Instead, herbal remedies necessitate time and consistent usage to reveal their benefits. In fact, their application must be ongoing **(Marwati & Amidi, 2018)**. Cheral® is an herbal medicine that combines two well-known medicinal plants: *C. longa* and *P. niruri*. Both herbs have been traditionally used in various systems of medicine, including Jamu, Ayurveda and traditional Chinese medicine, for their therapeutic properties. While numerous studies have explored Cheral®, limited research has been conducted on the profile of T lymphocytes and regulatory T cells following Cheral® administration. Furthermore, investigations into the impact of administering Cheral® to healthy individuals have never been carried out. This research aims to provide insights into the T lymphocyte profile (CD4, CD8, $CD4^+CD62L^+$, $CD8^+CD62L^+$) and $CD4^+CD25^+$ regulatory T cells in normal mice after the administration of Cheral® at specific doses.

MATERIAL AND METHODS

Description of experimental animals

In this research, 24 female rats (*Rattus norvegicus*) Wistar strain were employed and divided into four groups: the control group, dose 1 group, dose 2 group, and dose 3 group. These rats were sourced from Murine Farm, Singosari, Malang, Indonesia, and had a minimum body weight of 120 grams while being 6–8 weeks old. The experimental animals were in good health, exhibiting activity, bright eyes, straight and undamaged legs, thick, white, and smooth fur, and a normal gait. The rats were housed in plastic cages with wire covers and bedding made of husk (wood shavings), which was replaced every 2-3 days. They were provided with sufficient food and water **(Suprihatin *et al.*, 2019)**.

Research design

Wistar rats were divided into four treatment groups, each with five replications. Female Wistar rats received oral treatment for a duration of 90 days (Table 1). On the 91st day of treatment, the rats were sacrificed, and their spleen organs were excised. The spleen tissues were subsequently homogenized for antibody staining and flow cytometry analysis. All research procedures were subject to evaluation and approval by the Ethics Commission of Brawijaya University, Malang, Indonesia, under reference No: 021-KEP-UB-2023.

Administration of Cheral®

The Cheral® used in this study was obtained from PT. Ismut Fitomedika Indonesia (IFI), Makassar, Indonesia, in the form of powder. Cheral® is a product that contains a combination of Curcuma longa and Phyllanthus niruri with a product composition ratio of 1:1. Cheral® was weighed according to the specified dosage (Table 1). The dose is determined by converting the human dose into a rat dose by the Food and Drug Administration (FDA) provisions. The dose of Cheral® administration is 1500 mg daily (500 mg x 3 usage) for adults or 25 mg/Kg BB human. The human equivalent dose calculation based on body surface area from FDA guidelines for a human to a rat is 0.16 (**Jacob *et al.*, 2020**). Next, the converted dose from human to rat is calculated as 25: 0.16, resulting in 156.25 mg/Kg BW for the first dose (low dose). Each dose is reached up to 3 levels (Table 1). Furthermore, it was dissolved in sterile aquades. The Cheral® administration volume was 2 ml, given to rats with a body weight of 200 grams. The treatments were orally administered to all groups for 90 days.

Table 1 Treatment groups

Groups	Treatment Groups
Normal (N)	Administered distilled water (control)
Dose 1 (D1)	Administered Cheral® 156,25 mg/Kg BW
Dose 2 (D2)	Administered Cheral® 312,5 mg/Kg BW
Dose 3 (D3)	Administered Cheral® 468,75 mg/Kg BW

Isolation of spleen

On the 91st day, all rats that had received treatment for 90 days were sacrificed. The rats were injected with 0.1 ml of ketamine into the rat's femur. Ketamine is widely used as an anesthetic in mouse models for surgical procedures because of its effective analgesic and sedative properties. The rats were allowed to become immobilized. Subsequently, the spleen organ was isolated to obtain lymphocyte T cell. The spleen was selected because it is a secondary lymphoid organ with a high concentration of lymphocytes, including T cells. mature lymphocytes, such as B and T cells, encounter antigens and mount immune responses within the spleen. Consequently, this organ plays a crucial role in adaptive immunity (**Lewis *et al.*, 2019**).

Immunostaining dan flow cytometry

The isolated spleen was homogenized using the tip of a syringe in a dish containing PBS. The obtained suspension was transferred to a 15 mL polypropylene tube and supplemented with PBS to reach a volume of approximately ±10 mL. The suspension was then centrifuged at 2500 rpm, 10°C, for 5 minutes. The supernatant was remove, and the pellet was added with 1 mL of PBS. The resuspension was divided into microtubes containing 50μL, for antibody staining. The cells were extracellularly stained with 50 μL of FITC-conjugated anti-rat CD4 (Biolegend®, San Diego), FITC-conjugated anti-rat CD8 (Biolegend®, San Diego), PE/Cy5-conjugated anti-rat CD62L (Biolegend®, San Diego), PE/Cy5-conjugated anti-rat CD25 (Biolegend®, San Diego). The antibody staining procedure was carried out following by **Adharini *et al.* (2020)**.

Data analysis

The data obtained from flow cytometry were analyzed using BD Cellquest ProTM software (BD Biosciences, San Jose, CA, USA). The data flow cytometry was tabulated in Microsoft Excel for further statistical analysis using the SPSS software with a one-way analysis of variance (ANOVA). Subsequently, a Post hoc test was conducted with a significance level of p-value < 0.05 (**Adharini *et al.*, 2020**).

RESULTS AND DISCUSSION

Result

The study assessed the impact of administering Cheral® to female mice for a 90-day duration on the profiles of CD4 and CD8 T cells. The analysis results revealed variations in the profiles of CD4 T cells among the control group and those treated with three different doses of Cheral®, highlighting significant differences compared to the untreated controls. In the control group, the CD4 T cells profile exhibited an average cell count of 31.37%. Conversely, the administration of doses 2 (D2) Cheral® resulted in significantly higher CD4 profiles (36.10%) when compared to the control group. Moreover, the CD8 T cells profile in the control group displayed a value of 22.96%. The administration of dose 1 (D1) Cheral® had a significant effect on increasing the CD8 profile (28.52%) compared to the control group. Notably, Cheral® demonstrated significantly higher CD8 T cells profile values than the control group (refer to figure 1). The relative number of CD4 T cells was higher than that of CD8 T cells, indicating that the administration of Cheral® was capable of maintaining the healthy condition of the mice throughout the treatment period.

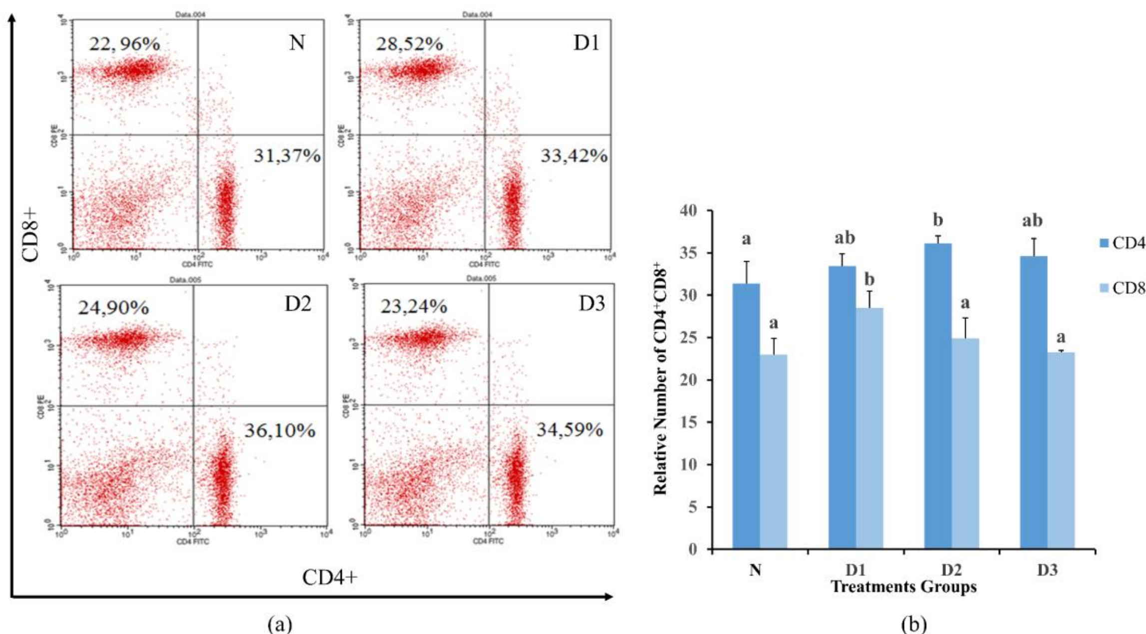

Figure 1 Profile of $CD4^+CD8^+$ in all treatments after 90 days treatment. (a) Spleen cells ($2x10^6$) contained in all treated rat models, then subjected to extracellular staining cells with anti-CD4 antibody and anti-CD8 antibody and analyzed using flow cytometry. N: Normal (control), D1: dose 1, D2: dose 2, D3: dose 3. (b) Statistical analyis of profile $CD4^+CD8^+$ in all treatments. The data are mean value ± SD of six rats in each group with a significant value p<0.05 (n = 24)

The findings from the analysis of $CD4^+CD62L^+$ T cells in both the control group and Cheral® treatment groups are presented in figure 2. Control female mice exhibited an average cell count of 18.93%. However, when Cheral® was administered at dose 1 (156.25 mg/kg BW) in the first group, it resulted in a significantly higher average cell count of 31.28%, marking a substantial difference from all other treatments. In the second group, dose 2, the average cell count was 22.02%, while in the third group, dose 3, it was 9.89%. Notably, the treatment at dose 3 recorded the lowest relative cell count compared to all other treatment

groups. Moreover, the evaluation of the CD8⁺CD62L⁺ T cells profile in control female mice indicated an average cell count of 57.82%. Notably, administering Cheral® at dose 1 yielded a significantly higher value compared to the control group and dose 3. Conversely, the administration of Cheral® at dose 3 resulted in a significantly lower average cell count than the other treatment groups. Specifically, the average cell counts for dose 1, dose 2, and dose 3 were 70.03%, 63.40%, and 47.47%, respectively.

The Treg cell profile after the administration of Cheral® did not yield significantly different results when compared to the control group (15.02%) (refer to figure 3). The relative numbers of Treg cells for dose 1, dose 2, and dose 3 were 16.32%, 16.85%, and 13.70%, respectively (figure 4). Notably, at dose 2, there was a relatively higher regulatory T cells profile compared to the control group, although the results were not statistically significant. This suggests that the administration of Cheral® does not have a noticeable impact on the Treg cell profile, as it does not induce a significant increase or decrease. Consequently, it can be assumed that Cheral® is capable of maintaining the Treg cell profile under normal conditions, similar to the healthy control group.

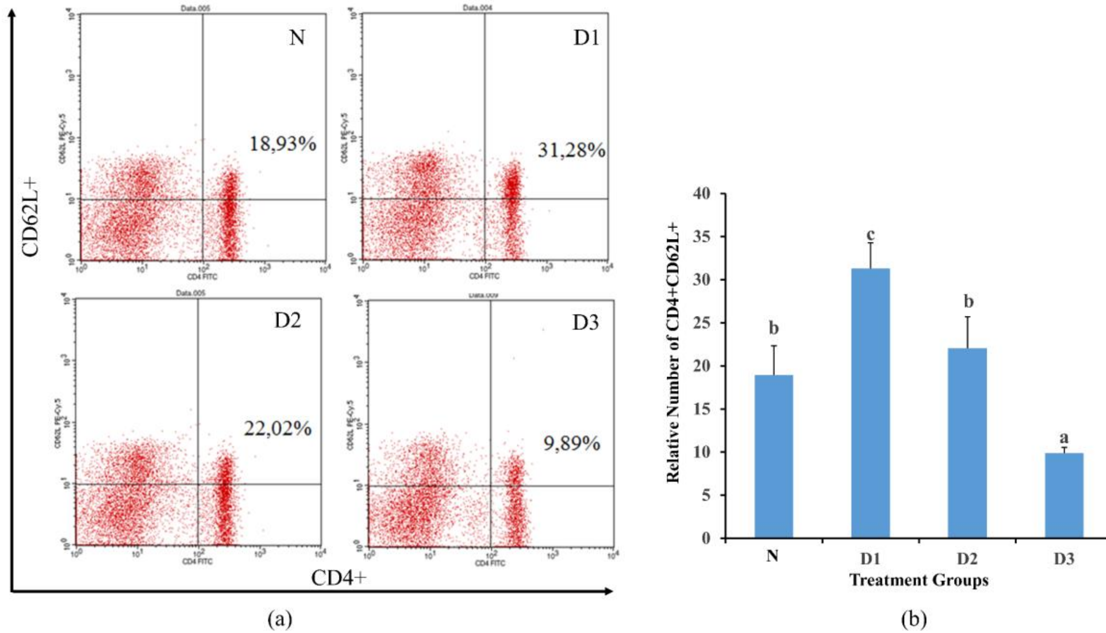

Figure 2 Profile of $CD4^+CD62L^+$ in all treatments after 90 days treatment. (a) Spleen cells ($2x10^6$) contained in all treated rat models, then subjected to extracellular staining cells with anti-CD4 antibody and anti-CD62L antibody and analyzed using flow cytometry. N: Normal (control), D1: dose 1, D2: dose 2, D3: dose 3. (b) Statistical analysis of profile $CD4^+CD62L^+$ in all treatments. The data are mean value ± SD of six rats in each group with a significant value $p<0.05$ (n = 24)

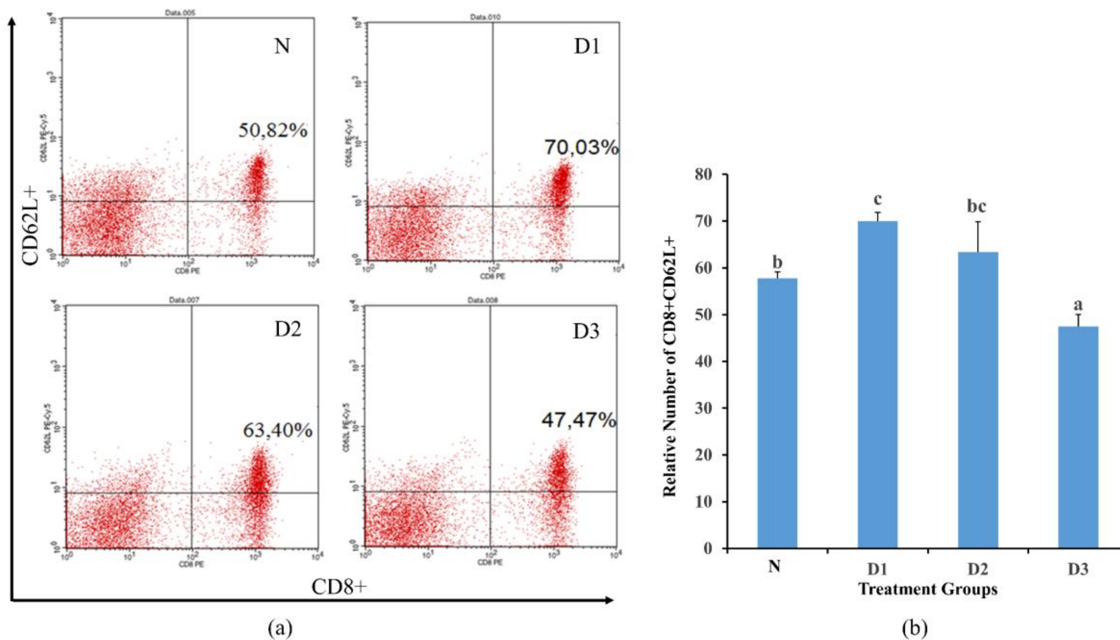

Figure 3 Profile of $CD8^+CD62L^+$ in all treatments after 90 days treatment. (a) Spleen cells ($2x10^6$) contained in all treated rat models, then subjected to extracellular staining cells with anti-CD8 antibody and anti-CD62L antibody and analyzed using flow cytometry. N: Normal (control), D1: dose 1, D2: dose 2, D3: dose 3. (b) Statistical analysis of profile $CD8^+CD62L^+$ in all treatments. The data are mean value ± SD of six rats in each group with a significant value $p<0.05$ (n = 24)

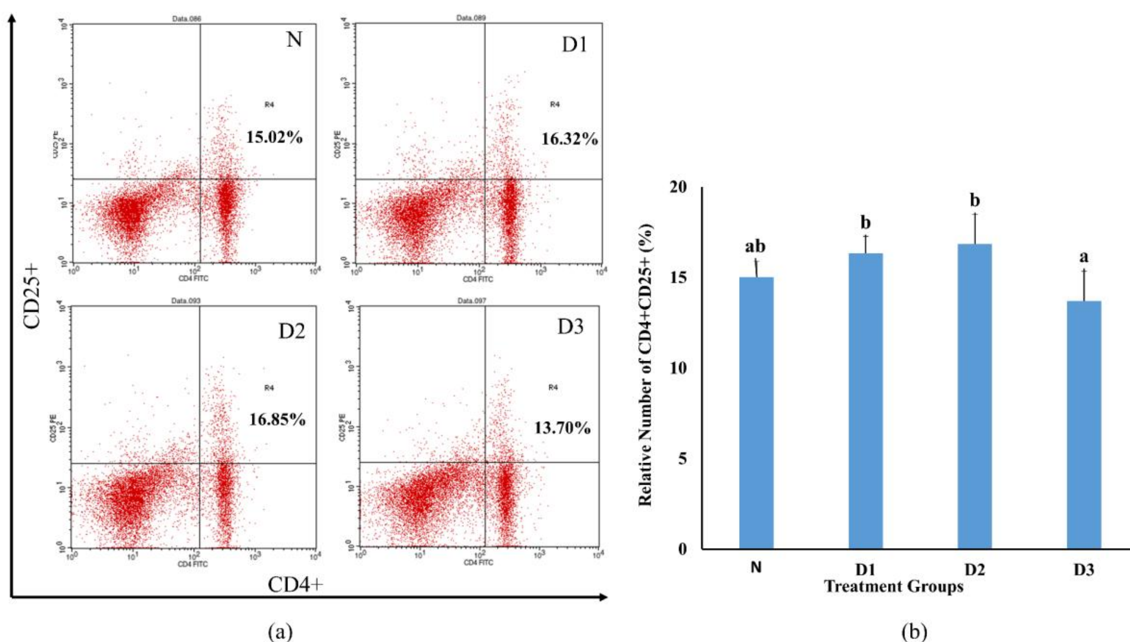

Figure 4 Profile of regulatory T cells ($CD4^+CD25^+$) in all treatments after 90 days treatment. (a) Spleen cells ($2x10^6$) contained in all treated rat models, then subjected to extracellular staining cells with anti-CD4 antibody and anti-CD25 antibody and analyzed using flow cytometry. N: Normal (control), D1: dose 1, D2: dose 2, D3: dose 3. (b) Statistical analysis of profile Regulatory T Cells ($CD4^+CD25^+$) in all treatments. The data are mean value ± SD of six rats in each group with a significant value $p<0.05$ (n = 24)

DISCUSSION

The administration of Cheral® was found to be non-toxic and effective in maintaining the health of the treated mice. *C. longa*, commonly known as turmeric, contains curcumin as its primary active compound. Curcumin has been extensively studied for its immunomodulatory effects, particularly on lymphocyte populations, including CD4+, CD8+, and regulatory T cells (Tregs) (Yuandani et al., 2021). *P. niruri* enhances immune function by activating lymphocytes, modulating cytokine levels, and providing antioxidant support, all of which are driven by its rich phytochemical profile (Wahyuni et al., 2023). Traditionally, both Phyllanthus niruri and Curcuma longa have been used in Indonesia to treat infections and support recovery from fevers, which aligns with their immune-boosting properties. Furthermore, the observed increase in the number of CD4 T cells suggests that the compounds present in Cheral® can support the body's immune system. CD4 T cells, a type of T helper cell, play a crucial role in orchestrating the immune response against foreign antigens **(Zhu & Zhu, 2020)**. An augmentation in the number of CD4 T cells may indicate that the compounds have a positive impact on immune system enhancement **(Oh & Fong, 2021)**. CD4 is a glycoprotein expressed on the surface of various immune cells, including T helper (Th) cells, monocytes, macrophages, and dendritic cells **(Chen *et al.*, 2022)**. CD4 plays a significant role in transmitting signals to other immune cells such as CD8 **(Li *et al.*, 2022)**. Several studies have demonstrated that administering plant extracts containing flavonoids can lead to an increase in the profiles of both CD4 and CD8 T cells in several types of disease such as tumor and cancer disease **(Sun *et al.*, 2022)**.
The profile of CD4 helps identify and destroy pathogens and signals other immune cells to respond to infections. Meanwhile, CD8 acts as a suppressor in the immune system **(Xie *et al*., 2023)**. The results showed that the relative number of CD4 cells was higher than CD8 cells (Figure 1), indicating better immune system health **(Taylor *et al*., 2020)**. Furthermore, increased CD8 cells inhibit excessive immune responses and terminate immune responses, which can help prevent disease progression. CD8 T cells are cytotoxic cells that can kill virus-infected or tumor cells. Increasing the number of CD8 T cells increases the body's ability to eliminate pathogens and abnormal cells effectively **(Sun *et al.*, 2023)**. CD8 T cells can respond more quickly to new infections or already-recognized antigens. Some activated CD8 T cells will differentiate into memory cells that stay in the body for a long time. These memory cells provide long-term protection and rapid response if the same pathogen re-infects **(Kalia *et al*., 2010)**.
The results showed a difference in the relative number of $CD62L^+CD4^+$ and $CD62L^+CD8^+$, which was higher after Cheral® administration compared with the control group (Figures 2 & 3). The high number of naive T cells (CD4 and CD8) allows the immune system to have a reserve of cells that can be activated in the face of new infections or vaccinations **(Taus *et al.,* 2022; Goronzy *et al.,* 2015).** CD62L or L-selectin is an adhesion molecule that functions by attaching and rolling along blood vessel endothelial cells. This molecule can be expressed by naive $CD4^+CD62L^+$ and $CD8^+CD62L^+$ T cells. Naive T cells circulate within the blood, spleen, and peripheral lymph nodes until they encounter an antigen. Upon encountering an antigen, naive T cells become activated and lose the CD62L molecule **(Tantalo *et al.*, 2021)**. The expression of CD62L can facilitate the migration of T cells into non-lymphoid tissues, including the skin and mucosa. T cells expressing CD62L can roll on endothelial cells and subsequently migrate into non-lymphoid tissues, such as the skin and mucosa, thereby enabling T cells to participate in immune responses to antigens located outside the lymphatic system **(Osborn *et al.,* 2019)**.
The expression of CD62L is used to assess the average population of CD8 and CD4, specifying their roles as cytotoxic and helper cells, respectively **(Lesteberg *et al*., 2023)**. The profile of $CD62L^+CD4^+$ cells is reserve cells for the body to respond to new antigens. At the same time, CD4 is a surface marker typical for helper T cells that regulate immune responses by assisting the activation of B cells to produce antibodies and helping the activation and function of cytotoxic T cells (CD8) and macrophages **(Kasuya *et al.,* 2024; Richards *et al.,* 2008)**. Naive CD8 T cells require activation through antigen recognition by Antigen Presenting Cells (APCs) via Major Histocompatibility Complex-1 (MHC-1) in secondary lymphoid organs **(Mortezaee & Majidpoor, 2022)**. Subsequently, the activated CD8 T cells undergo differentiation into a population of memory T cells that play a role in controlling cell localization and maintaining cell homeostasis **(Kok *et al.*, 2022)**. In healthy individuals, naive T cells express the CD62L molecule in more than 80% of cases. Conversely, in unhealthy individuals, naive T cells may experience a decrease in CD62L expression in peripheral lymph nodes, leading to T cell activation. The CD62L molecule then downregulates T cells, obstructing their entry into lymph nodes' endothelial cells. This, in turn, prevents the killing of APC dendritic cells due to the activation of CD8 T cells and leads to an increase in T cells that combat incoming antigens **(McRitchie & Akkaya, 2022)**.
Additionally, Treg cells play a central role in regulating inflammation and the immune system in various contexts, including the control of inflammatory responses through various effector mechanisms **(Shevyrev & Tereshchenko, 2020)**. Moreover, the profile of Treg cells did not exhibit significant differences when compared to the control group. This indicates that the administration of Cheral® does not disrupt the balance of Treg cell numbers within the immune system. Cheral® holds the potential to modulate the body's immune response without interfering with the crucial function of Treg cells, which are pivotal in maintaining immune homeostasis. Significantly altering Treg cell expression can trigger autoimmune reactions or excessive inflammation **(Eggenhuizen *et al.*, 2020)**. When Treg cells experience reduced expression or dysfunction, or when there are excessive fluctuations in their numbers, the body's immune system may behave uncontrollably and mount responses against its own tissues and organs. Treg cells represent a subset of CD4 T cells with the role of inhibiting excessive activation of effector T cells, such as CD4 T cells and CD8 T cells, contributing to immune tolerance maintenance **(Rocamora-Reverte *et al.*, 2021)**. Furthermore, Treg cells are instrumental in preventing autoimmune reactions and excessive inflammation while facilitating efficient immune system function. Treg cells engage in interactions with effector CD4 T cells (Th), which are helper T cells responsible for coordinating the immune response **(Wan *et al.*, 2020)**. Treg cells also possess the capability to inhibit the activation of effector CD8 T cells (Tc), which are cytotoxic T cells involved in responding to and eliminating cells infected by viruses or cancer cells **(Mittermüller *et al.*, 2023)**. This illustrates the

interconnected relationships between the profiles of CD4, CD8, and CD62L T cells with regulatory T cells.

Curcuma longa and *Phyllanthus niruri* are two examples of herbal remedies that combine various plant components. According to previous research, both of these plants contain various active compounds with roles as antidiabetic, antioxidant, immunomodulatory, and anticancer agents (**Hermanto *et al.*, 2020; Puspitarini *et al.*, 2019**). The inclusion of *Curcuma longa* and *Phyllanthus niruri* in Cheral®, as demonstrated by this study, has shown that Cheral® exhibits superior T cells activity compared to the control group in maintaining the health of rat during treatment. The bioactive compounds known as curcuminoids have gained recognition for their potential as immunomodulators, antioxidants, anti-inflammatory agents, and anticancer agents (**Yuandani *et al.*, 2021**). *P. niruri* is known to contain a variety of anticancer compounds, including phyllanthin, methyl brevifolincarboxylate, geraniin, gallic acid, corilagen, rutin, quercetin, and ellagic acid (**Silva *et al.*, 2021**). Additionally, compounds like phyllanthin, hypophyllanthin, and corilagin have shown potential in possessing immunomodulatory properties (**Chopade *et al.*, 2021**).

Additionally, the profile of regulatory T cells after Cheral® administration indicates no significant differences and possesses relative values similar to the control group. This suggests that Cheral® is effective in preserving the profile of regulatory T cells, which play a crucial role in immune system homeostasis. The pharmaceutical effect of Cheral® on lymphocyte T cells and regulatory T cells has the potential for the development of herbal medicine. Throughout the 90-day treatment period, Cheral® demonstrated its ability to sustain immune system homeostasis, as evidenced by the profiles of CD4, CD8, CD62L T cells, and regulatory T cells. Moreover, the utilization of natural products, such as Cheral®, as agents for treating various diseases can be attributed to their effectiveness and safety, as demonstrated by the findings of this study.

CONCLUSION

In conclusion, the percentage of the average number of CD T cells profiles was higher compared to that of CD8 T cells during the treatment. The administration of Cheral® effectively increased the relative numbers of $CD4^{+}CD62L^{+}$ T cells and $CD8^{+}CD62L^{+}$ T cells. Moreover, the relative number of Treg cells in the Cheral® treatment group did not significantly differ from the control group. These results indicate that the administration of Cheral® successfully maintained the health of the mice during the treatment period.

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