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Assessing the Immunomodulatory and Hepatoprotective Activities of Aqueous Tuber Extract of *Typhonium flagelliforme* (Lood) Blume in BALB/c Mouse

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Abstract

Immune cells play a very significant role in the body due to their capacity to preserve homeostasis. They function primarily in the quick elimination of potentially harmful substances from the system. There is a trend towards the increased utilization of the therapeutic potential of medicinal plants to treat a wide range of diseases through the regulation of immunomodulatory mechanisms. In this present work, our objective was to determine the extent to which *Typhonium flagelliforme*, also known as rodent tuber, has an immunomodulatory effect in vivo in BALB/c mice. Four treatment groups were used in this investigation on BALB/c mice, including the control group with each group receiving 0, 50, 100 and 500 mg/kg BW of the plant extract respectively. After fourteen days of treatment, the mice were sacrificed for further analysis. The spleen was isolated for flow cytometry study, and it was then stained with antibodies against CD4, CD8, CD4CD62L, and CD4CD25. The relative number of each immune cell subgroup to be monitored was calculated using the BD FACS Calibur flow cytometer. The liver was also subjected to histological examination using hematoxylin-eosin staining. Our findings indicated the increasing number of $CD8^+$ T cells and $CD4^+CD62L^+$ naive T cells in the spleen. Similarly, the relative number of $CD8^+$ T and $CD4^+$ T cells in thymus were elevated. This findings suggest that the *T. flagelliforme* extract (TFE) exerted the immunomodulatory activity which promote some certain of immune cells.

Keywords: Flow cytometry, immunomodulatory, rodent tuber, T cell, *Typhonium flagelliforme.*

1. Introduction

The immune system has been the main hotspot in biological and medical research in recent years, particularly during the current pandemic outbreak. It is a highly critical and complex system responsible for distinguishing the body's cells and other harmless material from foreign and particularly dangerous material, protecting the body against infections and foreign substances (Childs *et al*., 2019; Huntington and Gray, 2018). It is a delicate system composed of cells, chemicals, pathways, and tissues that interact to generate an immune response to prevent or eradicate infections as quickly as possible while leaving the body's cells unharmed (Horwitz *et al*., 2019; Nicholson, 2016).Because humans are always surrounded by hazardous pathogens, toxins, and even cancer cells, it constantly evolves while maintaining vigilance for any signs of invasion or an impending threat. This ability makes it the most crucial element of our species' survival throughout the evolutionary history (Childs *et al*., 2019; Hurst and Magiorkinis, 2019).

The target of immune cells is to is to swiftly destroy and eradicate any potentially dangerous materials and cells while also being able to control themselves to operate within a reasonable operating window (Nicholson, 2016). This capability, however, might be extremely harmful if it either puts the immune system into a hyperdrive condition where it attacks healthy cells and tissues or seriously undermines its capacity and capability, that lowers the body's state of defense and makes the individual more prone to diseases (Kitcharoensakkul and Cooper, 2020; Lotfi *et al*., 2019). Therefore, the capacity of immune cells to continuously self-regulate and interact with one another, mostly through a variety of cytokines, is of utmost importance. However, some of the regulation mechanisms are still incompletely understood (Cicchese *et al*., 2018; Tourkochristou *et al*., 2021). The immune system often steers clear of these extremities of immune response spectrum through a control mechanism known as immunological homeostasis (Huntington and Gray, 2018). Various regulatory components normally carry out this mechanism, both in innate and adaptive immune systems, such as regulatory T cells (Tregs), regulatory B cells (Bregs), M2-like macrophages, mesenchymal stromal cells (MSCs), myeloid-derived suppressor cells (MDSCs), and complement regulatory proteins (CRPs) (Cao *et al*., 2019; Carvajal *et al*., 2019; Papp *et al*., 2017; Tao *et al*., 2019). It

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involves maintaining a balanced response to protect against infection and disease while avoiding overreaction and autoimmune reactions. Disruptions in immune homeostasis can lead to autoimmune disorders and increased susceptibility to infection.

The regulation of homeostasis by the immune system is not only dependent on internal factors including types of transcription factors like FOXO and Myb (Dias *et al*., 2017; Zaiss and Coffer, 2018), but it is an intriguing mechanism which is also highly influenced by external factors such as diet (Tourkochristou *et al*., 2021). Numerous herbs and spices, especially their essential oils, have been researched for their potential as immunomodulators. They include quercetin, kaempferol, rutin, genistein, hesperidin, ascorbic acid, and menthol. They can suppress the synthesis of TNF-α, IL-1, IL-2, IL-6, IL-8, and IL-1β while also stimulating the proliferation of human peripheral blood mononuclear cells (PBMC) and the expression of IFN-γ (Lee *et al*., 2021; Putra and Rifa'i, 2019; Bian *et al*., 2019; Cheng *et al*., 2019; Xiao *et al*., 2018; Orhan *et al*., 2016). Most of the well-studied immunomodulatory compounds came from well-known sources, such as herbs like *Leptadenia pyrotechnica*, *Zingiber officinale*, *Curcuma longa*, *Mentha* × *piperita*, and some fruits like *Citrus limon* and *Muntingia calabura* (Amorim *et al*., 2016; Dash *et al*., 2018; Karthikeyan *et al*., 2021; Miles and Calder, 2021; Orhan *et al*., 2016; Sujono *et al*., 2020; Yuandani *et al*., 2021; Mahassni and Alsahafi, 2022). These sources are highly abundant and commercially available all over the globe. Other less well-known indigenous species are also expected to have a similar advantage as immunomodulators; *T. flagelliforme*, sometimes referred to as rodent tuber, is one of them. It is native to Indonesia and belongs to the Araceae family. It is known for its distinctive long, slender, and whip-like leaves and has been investigated for its potential anti-cancer properties (Laurent *et al*., 2015). Various studies suggest that the *T. flagelliforme* extract could inhibit the proliferation of breast cancer cells, increase the expression of p21 and caspase-3 on MCF-7 cells, and stimulate apoptotic pathways on P388 and NCI-H23 cells (Crystalia and Hillary, 2022; Lai *et al*., 2008; Maher *et al*., 2021; Mohan *et al*., 2011). Although *T. flagelliforme* has a long history of use in traditional medicine, more research is needed to fully understand the mechanisms of action and potential therapeutic uses of the plant and its bioactive components. In this study, we examine the effect of rodent tuber extract in increasing the immune response as measured by the number of $CD4^+$, $CD8^+$, $CD4^+CD62L^+$, and $CD4^+CD25^+$ cells.

2. Materials and Methods

2.1. Experimental Animals Preparation

The mice (*Mus musculus*) utilized in this study were BALB/c strain, 8 weeks old, and in good health (active movement, no hair loss, no structural anomalies). They were then given food and water, *ad libitum*, and acclimatized for 7 days. The use of experimental animals has received an ethical certificate No. 72-KEP-UB from the Brawijaya University Ethics Committee. This study included four treatments and three replications for a total of twelve mice.

2.2. Water Fraction of Rodent Tuber Preparation and Injection on Mice

The rodent tuber suspension was made by diluting rodent tuber extract powder with water. The rodent tuber powder was obtained by cutting the roots into little pieces, drying them in direct sunlight, and then grounded until they become powder. Meanwhile, the powdered roots are mixed with water according to the dose injected to produce herbal suspensions. The prepared suspension was administered orally to mice twice daily for 14 days, at doses of up to 50 mg/kg BW in treatment group I, 100 mg/kg BW in treatment group II, and 500 mg/kg BW in treatment group III.

2.3. Spleen Isolation Procedure

The isolation procedure was carried out in accordance with our previous studies (Putra *et al.*, 2021; Putra *et al*., 2016). The spleen was separated from the dissected organs of the mice, washed in sterile PBS and filtered using a 100µm BD nylon cell strainer. Then, it was homogenized by compressing it with the syringe's base in one direction from top to bottom, about 2-3 times, until the suspension could be filtered in a petri dish. The filtered suspension was placed in a sterile microtube with sterile PBS and then centrifuged for 5 minutes at 4^{o}C at 1500 rpm. The supernatant was discarded, and the pellet was resuspended and homogenized with 100-1000µl PBS. The suspension was repeatedly centrifuged until it formed a white pellet.

2.4. Cells Quantitative Analysis using Flow cytometry

The pellet was resuspended in 1 ml of sterile PBS. A 100µl homogenate was transferred to a fresh microtube. It was centrifuged again for three minutes at 2500 rpm and 4°C, and the supernatant was discarded. Anti-$CD4^+$, anti-$CD8^+$, anti-$CD62L^+$ and anti-$CD25^+$ antimouse antibodies were then applied to the pellets. The flow cytometer cuvette was loaded with the sample and 400 µl of sterile PBS was added. The cuvette was connected to the BD FACS Calibur flow cytometer nozzle. The BD Cell Quest Pro software was used for the analysis, set in acquiring mode (Putra *et al.*, 2020; Putra *et al*., 2015).

2.5. Liver Slide Preparations and Histopathological Observations

The histopathological examination was conducted according to the previous study with few minor adjustments (Putra and Rifa'i, 2020; Putra *et al.*, 2017). The liver was isolated from dissected mice and then cleansed of any remaining blood with PBS before being placed in a 4% PFA fixative solution in PBS at room temperature for 1–7 days. The paraffin technique was used to prepare the liver slides. To remove the paraffin, the preparations were deparaffinized with xylol twice for 4 minutes each and then soaked with graded alcohol (100%, 95%, 90%, 80%, 70%, 60%, 50%, and 30%) for 3 minutes each. After washing with distilled water for 5 minutes, the slides were stained with hematoxylin for 1 minute, rinsed for 5 minutes with distilled water, and submerged in graded ethanol (30%, 60%, 70%) each for 5 minutes. After 10 minutes of eosin, it was rehydrated in graded ethanol (70%, 60%, 30%) for 5 minutes each, cleaned with xylol for 3×5 minutes, and mounted with entellan^{TM}. An Olympus BX51 microscope coupled with an Olympus DP20 digital camera was used to make the observations.

2.6. Observation Parameter

This study investigated both qualitative and quantitative factors. In control and treated animals, changes in the number of $CD4^+$, $CD8^+$, $CD4^+CD62L^+$, and $CD4^+CD25^+$ cells were evaluated quantitatively using flow cytometry (Figure 1). The impact of injecting rodent tuber extract on the histological microstructure of the liver with hematoxylin-eosin staining was the qualitative parameter evaluated using a light microscope. The existence or absence of damage to the liver as an antitoxic organ was the focus of the observations. The presence of bleeding, congestion, and necrosis of the hepatocytes following the treatments was used to determine the degree of liver injury.

2.7. Data Analysis

This study employed a completely randomized design (CRD) and the 95% confidence interval ANOVA test. The data was collected in changes in the number of $CD4^+$, $CD8^+$, $CD4^+CD62L^+$, and $CD4^+CD25^+$ cells, which were statistically assessed using normality and variance homogeneity tests. A two-way ANOVA with α=0.05 significance level was used to evaluate normally distributed data with homogenous variance. If $p<0.05$ indicates a substantial difference between the tested treatments and vice versa. The Tukey HSD test was then used as a post-hoc test. All of the statistical analysis was carried out by SPSS 16.0 for Windows.

3. Results and Discussion

3.1. The Analysis of the Relative Number of $CD4^+$ and $CD8^+$ Cells in Thymus

The flow cytometry results of the relative amount of $CD4^+$ cells on the thymus are shown in figure 2. The TFE100 group exhibited a significant increase in the relative number of $CD4^+$ T cells at 29.10%. It was significantly higher than the TFE50 group at 10.64%. Meanwhile, the flow cytometry results on the relative amount of $CD8^+$ cells on the thymus are shown in figure 2. The TFE500 group had a cell count of 3.72%, while the TFE50 group had about 2.79%. Both are statistically insignificant compared to the normal group at 2.05%. However, the TFE100 group's results show a different trajectory by significantly increasing the $CD8^+$ cell number to 14.14%.

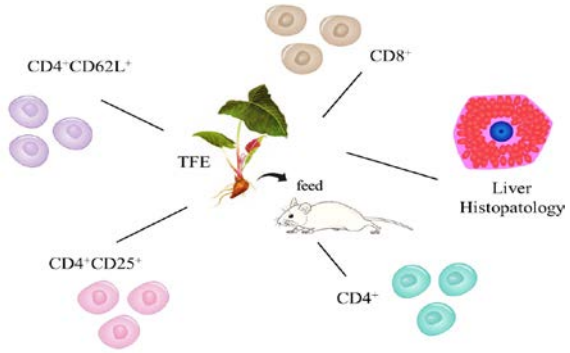

Figure 1. Major immune cell subsets assessed following TFE treatment.

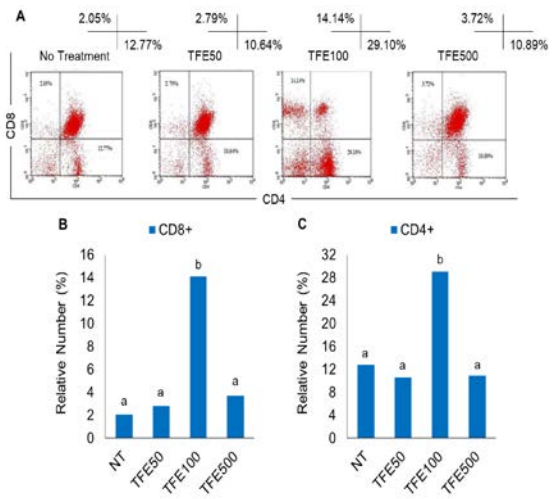

Figure 2. Immunomodulatory assessment of the effect of TFE on CD8 and CD4 T cells in thymus. (A). Flow cytometry graph of $CD8^+/CD4^+$; (B). Bar graph of $CD8^+$; and (C). Bar graph of $CD4^+$. The different alphabets indicate statistical significance compared to the other groups with p-values < 0.05.

The increased relative number of naïve $CD4^+$and $CD8^+$cells could be attributed to the content of various fatty acids, including oleic acid, palmitic acid, butyric acid, linoleic acid, 9-hexadecanoic acid, cis-13-octadecenoic acid, and stigmasterol (Lai *et al*., 2010; Mohan *et al*., 2011; Sianipar *et al*., 2016; Sianipar *et al*., 2019). Accordingly, a study has shown that *T. flagelliforme* contains two rare fatty acids namely benzenetridecanoic acid and benzenetridecanoic acid methyl ester (Chen et al., 1997). This suggest *T. flagelliforme* might exert the immunomodulatory activity by interfering the number of naïve $CD4^+$ and $CD8^+$ cells through its active metabolites. However, up-to-date, the molecular pathway by which those fatty acids affect the number of naive $CD4^+$ and $CD8^+$ T cells in the thymus is not fully understood (Hidalgo *et al*., 2021).The thymocytes migrate from bone marrow to the thymus to undergo the maturational stages. They undergo several stages until the culminating stages become single positive for either CD4 or CD8 (Mothe-Satney *et al*., 2016).

During those processes, we suggest that one of the possible ways oleic acid, linoleic acid, and other unsaturated fatty acids may increase the number of naive $CD4^+$ and $CD8^+$ T cells in the thymus is by increasing their survival rate during the thymic selection process. However, its exact mechanism is still highly unclear. One of our suspected pathways involved is the peroxisome proliferator-activated receptor (PPAR) pathway because it is activated by fatty acids including oleic acid and linoleic acid, and regulates the T cells' survival during its developmental process in the thymus (Le Menn and Neels, 2018; Mothe-Satney *et al*., 2016). Those studies, however, suggested opposite results. The activation of PPARβ decreases the proliferation rate of CD4−CD8− double-negative stage 4 thymocytes caused by increasing fatty acid oxidation. Other in vitro studies suggest that those unsaturated fatty acids could interfere with T cells' signal transduction before any antigen stimulation, resulting in reduced proliferation capabilities of naïve T cells. In high doses, oleic acid could induce apoptosis by activating the

caspase-3 pathway (Radzikowska *et al*., 2019; Reilly *et al*., 2021).

3.2. The Analysis of the Relative Number of CD4$^+$ and CD8$^+$ Cells on Spleen

We examined the body's immunological response to rodent tuber extract administration and investigated the association between multi-dose dosages and changes in the relative numbers of CD4$^+$ and CD8$^+$ T cells in the mice. As the proportions of CD4$^+$ and CD8$^+$ T cell counts in the spleen were calculated using flow cytometry, there was a substantial shift in the relative number of CD4$^+$ and CD8$^+$ cells in all dosage treatments compared to the control. The results in figure 3 depict the proportion of the relative amount of CD4$^+$ T cells in each treatment dosage. The relative number of CD4$^+$ T cells in the control group was 18.63%.The relative number of CD4$^+$ T cells in TFE50was increased by 1.33% to 19.96%, although the increase was statistically insignificant. Meanwhile, the relative number of CD4$^+$ T cells reduced significantly to 12.3% at TFE100 and 11.065% at TFE500. On the other hand, the same flow cytometry results showed an increase in the number of CD8$^+$ T cells, with 11.79%, 9.25%, and 10.7% for TFE50, 100, and 500 groups, respectively, compared to the control group with 7.37%.

With increasing treatment doses, fewer CD4$^+$ T cells were present; however, more CD8$^+$T cells were prevalent. The first one could appear counterintuitive because it can result in immunodeficiency. We have not been able to come up with a reasonable explanation for whether *T. flagelliforme* extract's bioactive compounds would have this impact because of limited studies on the medicinal plants, and given that it is frequently brought on by liver-related dysregulation of lipid metabolism (Tran and Sitia, 2016). However, a study found that linoleic and oleic acids could cause murine CD4$^+$ T cells to undergo apoptosis, slow down their proliferation rate, and block the T cells' activation and differentiation. Particularly oleic acid inhibits the expression of IL-2 and IFN-γ (Hidalgo *et al*. 2021). The increased number of apoptotic cells may be brought on by PPARα activation, which causes an increase in carnitine palmitoyltransferase (CPT) on the mitochondrial membrane and impairs the function of the electron transport chain. One of the critical properties of CD4$^+$ T cells is that they have more mitochondria than CD8$^+$ T cells, which makes them more susceptible to oxidative stress brought on by the production of ROS, which increases the rate of apoptosis(Brown *et al*., 2018; Reilly *et al*., 2021). On the other hand, phytol also decreases the number of CD4$^+$ T cells, particularly Th1 cells, through NOX2-induced ROS generation (Blum *et al*., 2018).

The number of CD8$^+$ T cells has been seen to rise with increasing treatment doses, in contrast to its counterpart. The concern is that not each of the major bioactive components in *T. flagelliforme* extract has been properly examined for its impact on the number of CD8$^+$ T cells in the spleen. A study suggests that *T. flagelliforme* extract could increase the number of CD8$^+$ T cells in immunocompromised mice after cyclophosphamide induction (Nurrochmad *et al*., 2015). According to one study on autoimmune uveitis, phytol may enhance the CD8$^+$ T effector/memory cells, a particular subset of CD8$^+$ T cells, in the spleen (Daudin *et al*. 2011). However, according to another study, neither palmitic acid, linoleic acid, nor oleic acid have any real impact on the population of cells (Medrano *et al*., 2022). Other research suggests that palmitic acid may reduce CD8$^+$ T cell numbers by interfering with mitochondrial function (Manzo *et al*., 2020).

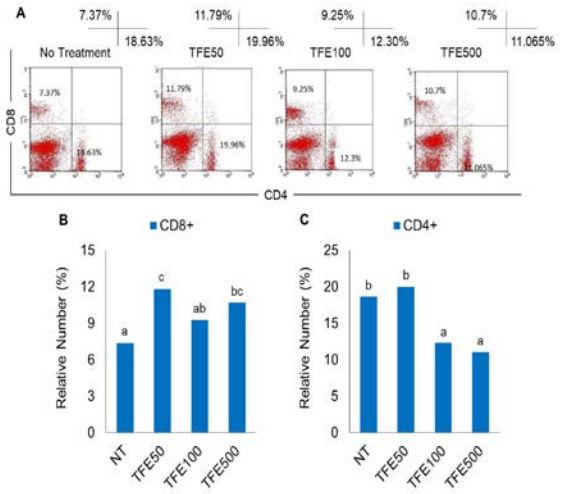

Figure 3. Immunomodulatory assessment of the effect of TFE on CD8 and CD4 T cells in spleen. (A). Flow cytometry graph of CD8$^+$/CD4$^+$; (B). Bar graph of CD8$^+$; and (C). Bar graph of CD4$^+$. The different alphabets indicate statistical significance compared to the other groups with p-values < 0.05.

The CD4$^+$/CD8$^+$ ratio, on the other hand, revealed that the ratios for the control, TFE50, 100, and 500 groups, respectively, were 2.520, 1.693, 1.330, and 1.034. The CD4/CD8 ratio refers to the relative number of CD4$^+$ T cells and CD8$^+$ T cells, including their respective subsets in the immune system (Golubovskaya and Wu, 2016). It is a crucial factor in determining whether the immune function has been altered. It also serves as a marker for chronic inflammation, particularly that brought on by HIV(Aiello *et al*., 2019; McBride and Striker, 2017). It is still unclear what the precise CD4$^+$/CD8$^+$ ratio normal range should be. However, several research showed the acceptable normal ratio, higher than 1.0(McBride and Striker, 2017; Tang *et al*., 2015). The control group in this study exhibited the highest ratio, yet as the treatment dose increased, the ratio plummeted to virtually 1.0, which did not progress as we expected.

3.3. The Flow Cytometry Analysis of the Relative Number of CD4$^+$CD62L$^+$ Cells

The flow cytometry results of CD4$^+$CD62L$^+$ T cells revealed a substantial reduction in the relative number of CD4$^+$CD62L$^+$ T cells in TFE50 and TFE100 compared to the control (Figure 4). The relative number of CD4$^+$CD62L$^+$ T cells in the TFE50 group was down to 3.55%, the TFE100 group was down to 5.78% after the oral treatment, while the control group was maintained at 7.67%.

Our chosen markers are meant to identify the naive T helper cell population, which frequently expresses both markers but more significantly, CD62L, a cell adhesion marker that aids the naive cells in homing to secondary lymphoid organs (Putra *et al*., 2023; Rahayu et al., 2022; Watson *et al*., 2019; Sckisel *et al*., 2017; Yang *et al*., 2011).

Previously, we made the assertion that the influence of the extract's predominant bioactive constituents, unsaturated fatty acids, on the population of naive T helper cells is yet unclear. However, the second conceivable reason for this phenomenon may be related to unsaturated fatty acids' capacity to affect CD62L expression. According to a study, unsaturated fatty acids may inhibit its expression on murine naive T helper cells (Anderson and Fritsche, 2004) making it undetectable by the designated antibody. While we are still looking for an explanation for this occurrence, it is hypothesized that it is related to the Akt pathway's activation by linoleic and oleic acids, which suppress CD62L expression (Crompton *et al*., 2015; Marcial-Medina *et al*., 2019; Serna-Marquez *et al*., 2017). It might have an impact on how naive T helper cells are conveyed. However, the same publication makes the case that adding polyunsaturated fatty acids may assist naive T helper cells survive in the short term in the absence of immunological activation (Anderson and Fritsche, 2004). Conversely, it has been demonstrated that saturated fatty acids, such as palmitic acid, can encourage the development of effector T cells into a proinflammatory subset by upregulating the expression of SLAMF3 and IFN-γ, but this impact was seen when mice were fed a high-fat diet (Zhou *et al*., 2019). Another hypothesis is that the phytol in the extract decreased the proportion of T cells expressing $CD4^+CD62L^+$ because it stimulated the activation of PPARα, a crucial regulator of IL-4 release and a catalyst for the differentiation of naive T helper cells into type 2 T helper cells (Th2) (Choi and Bothwell, 2012; Gloerich *et al*., 2005; Lai *et al*., 2008).

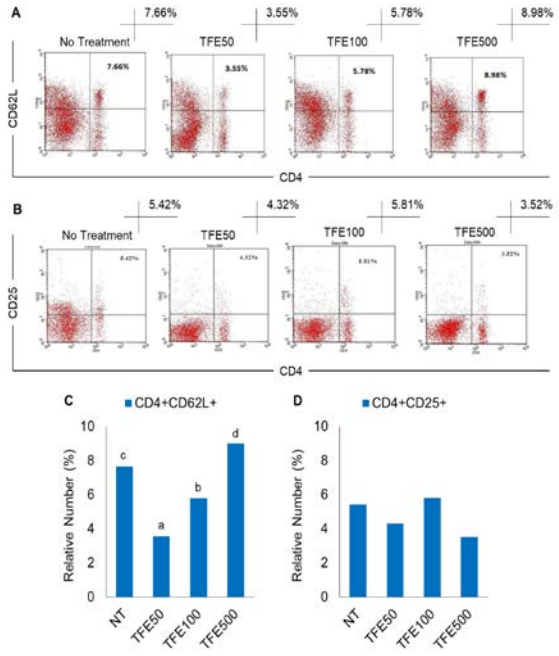

Figure 4. Immunomodulatory assessment of the effect of TFE on CD4CD62L naïve T cell and CD4CD25 regulatory T cell in spleen. (A). Flow cytometry graph of $CD4^+CD62L^+$; (B). Flow cytometry graph of $CD4^+CD25^+$; (C). Bar graph of $CD4^+CD62L^+$; and (D). Bar graph of $CD4^+CD25^+$. The different alphabets indicate statistical significance compared to the other groups with p-values < 0.05.

In contrast, providing the rodent tuber extract at a dosage of 500 mg/kg BW resulted in a substantial rise in the relative number of $CD4^+CD62L^+$ cells to 8.985% as opposed to a decrease when compared to the control. Unfortunately, given the kind and quantity of bioactive chemicals in the extract, we are unable to come up with a solid explanation for this behavior. It is suggested that the treatments reduced the number of T cells expressing $CD4^+CD62L^+$ because the naïve T cells were promoted into effector cells, such as $CD4^+$ T cells expressing $CD8^+$, $CD69^+$, $CD25^+$, and $CD44^+$. A reduction in the number of $CD4^+CD62L^+$ T cells reflects the activity of naïve cells, which transform into a subset of $CD4^+$ T cells, such as regulatory T cells, as a result of antigen exposure.

3.4. The Analysis of the Relative and Absolute Number of $CD4^+CD25^+$ Cells

The spleen flow cytometry results in figure 4 showed the relative number of $CD4^+CD25^+$ T cells among the total lymphocyte cells. It was demonstrated that mice treated with TFE100significantly increased $CD4^+CD25^+$ cells' numberto 5.81% compared to the control group with 5.42%, while the TFE500 group's cell number exhibited a significant drop to 4.32%. The most significant reduction in $CD4^+CD25^+$ cell number was observed inthe TFE50 group, which dropped as low as 3.53% (Figure 4).

One of the possible explanations for the low number of $CD4^+CD25^+$ T cells, better known as regulatory T cells (Tregs), was that the mice models were unexposed to any form of infections or antigens. Because of the nature of the study, which did not expose any antigen to the mouse model; we assumed that the Tregs population measured is categorized under naturally occurring Treg cells. Normally, the population number of naturally occuring Treg cells in the spleen for both human and murine is around 5-10% (Ali and Rosenblum, 2017; Lourenço and La Cava, 2011), which is importantly critical for modulating peripheral tolerance and preventing autoimmune disease (Rocamora-Reverte *et al*. 2021). This number, however, indicates that the population of $CD4^+CD25^+$ T cells in this study is in the lower band of the normal range. Nonetheless, autoimmune research suggests that the normal percentage range of circulating $CD4^+CD25^+$ T cells is from 0.6% to 7.9% (Nurrochmad *et al*., 2015), making the results completely in the acceptable range.

A study suggests that supplementing polyunsaturated fatty acid could increase the number of Tregs because PPARγ has a higher affinity for it (Kurniawan *et al*., 2020). Also, short-chain fatty acids such as butyric acid could induce the expression of FOXP3 by increasing the acetylation of histone H3 in the promoter region and promoting Treg formation (Kempkes *et al*., 2019). Oleic acid and 9-hexadecenoic acidare suggested to increase Tregs population number through promoting the expression of FOXP3 (Passos *et al*., 2016; Pompura *et al*., 2021). We found that almost no study depicted a decreased number of Tregs caused by bioactive compounds in *T. flagelliforme* extract, shown at TFE50 and TFE500 administration. However, it is important to note that the research on their effect on Tregs is still in the early stages, and more studies are needed to fully understand the mechanisms underlying this effect and determine whether similar effects can be observed in humans.

We predominantly suggest that the subset of T cells we studied is Tregs, or naturally occurring Tregs, to be exact, because we assumed that it also expressed $FOXP3^+$.

However, it is worth noting that the $CD4^+CD25^+$ T cells could behave as effector cells in addition to being regulatory cells because they lackFOXP3 protein ($CD4^+CD25^+FOXP3^-$), which is a determinant of their activity as regulators. They were conventional cells activated to become effector cells after exposure to allergens (Wing *et al*., 2002).Further research is needed to precisely determine the exact function of $CD4^+CD25^+$ T cells in this study because $CD4^+$ T cells expressing CD25 surface marker can serve as either conventional cells triggered by allergens or regulatory T cells. As a result, the involvement of $CD4^+CD25^+$ cells must be explored in future research focused on FOXP3. Because this study did not utilize an anti-Foxp3 antibody, the role of $CD4^+$ $CD25^+$ T cells in the results cannot be simply justified.

3.5. Liver Toxicity Analysis After Rodent Tuber Extract Administration

The cytotoxic impact of the rodent tuber extract on liver histopathology with hematoxylin-eosin staining revealed that the control group has hexagonal hepatocytes with a single nucleus, but some have two nuclei (binucleate) in its center. A cell's nucleus undergoing karyorrhexis may be noticed in the liver of TFE50 treatment mice (Figure 5). Administration of TFE100 and TFE500 might induce liver injury by necrosis (marked by an arrow) during the karyorrhexis stage, where nuclear cells in hepatocytes are fragmented or undergo cell lysis. Necrosis is a manifestation of liver damage caused by toxic compounds, includinghigh-level of saponins, or induced by certain diseases caused by viruses and bacteria. This term could be further categorized as drug-induced liver injury (DILI), a common side effect of all drug supplementations, including herbal, particularly as an effect of inhibiting critical enzymes, such as cytochromes and alanine aminotransferase (Mega *et al*., 2021; Navarro *et al*., 2017). The results found no statistically significant difference between the treatment groups, implying that all doses did not exert any harmful effect on hepatocytes nor trigger necrosis.

Although we cannot find plenty of research mentioned about the side effect of *T. flagelliforme* extract administration, we found research that has contradicting results, stating that it could have toxic effects on hepatocytes, defined by increased levels of Serum Glutamate Oxaloacetate Transferase (SGOT) and Serum Glutamate Pyruvate Transferase (SGPT) in the blood caused by damaged cells (Isturiningrum, 2010; Linasari, 2010). These effects are predicted to be an effect of high-concentration exposure to saponins because they could disrupt the integrity of the liver cell membrane. Saponins have been shown to have detergent-like properties, which can disrupt the lipid bilayer of the cell membrane. This phenomenon can cause leakage of intracellular contents and ultimately lead to cell death (Sudji *et al*., 2015). The detergent-like properties of saponins are thought to be due to their structural features, specifically the presence of a hydrophobic triterpene or steroidal sapogenin and a hydrophilic carbohydrate moiety (Mugford and Osbourn, 2012; Xu and Yu, 2021). The hydrophobic region of the saponin molecule can interact with the lipid bilayer of the cell membrane, disrupting its integrity and leading to cell death. In addition, saponins may also contribute to liver cell death by altering the absorption and metabolism of other substances. Saponins have been shown to interfere with the absorption of nutrients, particularly fat-soluble vitamins, which can lead to deficiencies that may contribute to liver injury and cell death (Pathaw et al., 2022; Samtiya et al., 2020). They may also alter the metabolism of certain drugs, potentially leading to liver injury and cell death. However, because we cannot identify any significant hepatocyte damage, it is safe to assume that the saponin content in the tested extract was well below the critical limit because a low concentration of saponins could have a hepatoprotective effect (Juszczak et al., 2021; Qu et al., 2012). Other than that, we cannot find any data on any toxic effect on hepatocytes caused by oleic acid, palmitic acid, butyric acid, linoleic acid, 9-hexadecanoic acid, cis-13-octadecenoic acid, and stigmasterol at possible concentration contained in the *T. flagelliforme* extract.

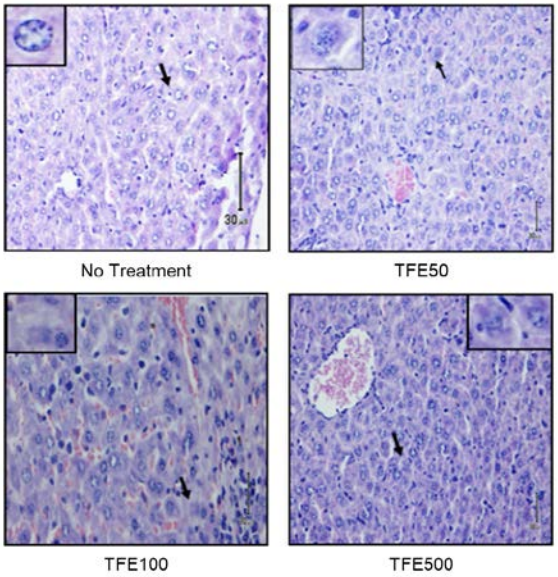

Figure 5. Representative microphotograph of liver section from experimental mice model (HE staining, M = 400×).

Although the common perception towards medicinal plants have far less concerning side effects than its commercial counterparts, it is not fully risk-free either. One of the most utilized herbals, turmeric, is thought to be the main cause of acute liver injury, particularly when combined with black pepper, because piperine in the black pepper increases the curcumin absorption up to 20 fold, causing hepatotoxicity effect (Halegoua-DeMarzio *et al*.,2022; Sohal *et al*., 2021).

T. flagelliforme extract, in addition to the immune cells mentioned previously, may influence other types of immune cells, according to various studies. In immunocompromised mice, the extract could increase the levels of various cytokines such as TNF-α and IL-1α, as well as the number and activity of macrophages, particularly their phagocytic index and capacity. However, in higher doses, the effect could also be reversed (Nurrochmad *et al*., 2015; Sagala and Murwanti, 2020). It also upregulates the heat shock protein-70 (Hsp-70) in the tumor associated macrophages, which then change their polarization towards pro-inflammatory M1-like macrophages, promoting cancer cell apoptosis via an extrinsic apoptotic pathway (Ibrahim *et al*., 2022; Lai *et al*., 2008). *T. flagelliforme* extract has been studied for its apoptosis activity or anti-proliferative effects on numerous of cancer cells (Table 1). For example, in the WEHI-3 leukemia model case, the extract administration also impacted the number of peripheral immature granulocytes

and monocytes by lowering their numbers (Mohan *et al*., 2010).

Overall, the data point to immune-modulating properties of *T. flagelliforme* extract, which may be helpful in the treatment of a range of immune-related conditions. To cover its effects on a wider spectrum of immune cells and completely comprehend the processes underlying these effects, further research is required due to its limited availability.

Table 1. Several distinct biological activities attributed to *T. flagelliforme* have been observed

No.	Experimental target	Biological activity	Reference
1	CSCC cells	↑ antiproliferation activity	Priosoeryanto et al., 2020
	Rabbit endothelial cells	↑ antiangiogenesis activity	
2	DMBA-Induced Rats Breast Tumor	↑ Cancer chemo-prepentive effect ↓ tumor incidence, tumor size, and tumor weight	Maysarah et al., 2020
3	RBL-2H3 cells	↑ anti-allergic activity	Korinek et al., 2017
4	HeLa and T47D cells	↓ telomerase expression	Purwaningsih et al., 2016
5	WiDr cells	↑ apoptosis activity ↓ COX-2 expression	Setiawati et al., 2016
6	CEMss cells	↑ cytotoxicity ↑ cellular DNA breaks ↑ apoptosis activity	Mohan et al., 2011
7	WEHI-3 cells BALB/c leukemia mice model	↓ proliferation activity ↓ immature granulocytes and monocytes	Mohan et al., 2010
8	CEMss cells	↑ apoptosis via ↑ activation of caspase-9, PARP cleavage and cytochrome c release	Mohanet al., 2010
9	NCI-H23 cells	↓ cancer cell growth ↑ induces apoptosis	Lai et al., 2008

4. Conclusion

Based on our findings, there was an increase in the number of $CD8^+$ T cells as well as $CD4^+CD62L^+$ naïve T cells in the spleen. Additionally, there was an increase in the relative number of $CD8^+$ T cells and $CD4^+$ T cells in the thymus. Based on these observations, it seems likely that TFE are responsible for the immunomodulatory function that helps particular immune cells thrive.

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Conflict of Interest

No conflict of interests.

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