DOI: 10.24996/ijs.2025.66.6.4

ISSN: 0067-2904

Evaluating the Immunomodulation and Hepatoprotective Activities of *Hydnophytum formicarum* Jack (Ant Plant) Tuber Extract in BALB/c Mouse

Wira Eka Putra[1], Anny Af'idaturrohmah[2], Arief Hidayatullah[3], Muhaimin Rifa'i[2,*]

[1]*Biotechnology Study Program, Department of Applied Sciences, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, East Java, Indonesia*

[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, East Java, Indonesia*

[3]*United Nations Development Programme Indonesia, Health Governance Initiative, Eijkman-RSCM Building, Jakarta, Indonesia.*

Received: 23/10/2023 Accepted: 10/6/2024 Published: 30/6/2025

Abstract

Contemporary developments have indicated an escalating demand for plant-derived pharmaceutical products. Apart from their economic value, the utilization of medicinal plants is believed to pose a considerably reduced risk of adverse effects when compared to the use of synthetic drugs. One of the approaches to determine the potential of a medicinal plant against biological diseases is the quest for novel medications using natural constituents. In this present investigation, we sought to determine the immunomodulatory and hepatoprotective properties of *Hydnophytum formicarum* in BALB/c mice. The study used 6-week-old healthy BALB/c mice. The treatment consisted of giving different doses of *H. formicarum* tuber extract. To observe the immunomodulation activity, we compared the relative numbers of various immune cell subsets, such as CD4, CD8, CD4CD25, and B220 cells. Furthermore, hematoxylin and eosin staining were conducted to see how the *H. formicarum* extract affected liver damage. The results showed that the *H. formicarum* affects $CD8^+$, $CD4^+$, $CD4^+CD25^+$, and $B220^+$ cells in a few spots. Importantly, the histopathological view showed that there were no significant differences between the control group and the treatment group. These results indicate that the *H. fomicarum* tuber extract did not harm the liver. Finally, to understand the role of *H. fomicarum*

*Email: rifa123@ub.ac.id

tuber as immunomodulator, comprehensive studies on other specific immune cells is necessary.

Keywords: Ant-plant tuber, diseases, *Hydnophytum formicarum,* mmunomodulatory, medicinal plant.

Introduction

Ethnomedicine, especially herbal medicine, has been integral to traditional healthcare systems for thousands of years, providing natural and effective remedies for various diseases and conditions [1, 2]. With increasing concerns over the side effects and toxicity associated with modern commercial drugs, there has been a growing interest in exploring the potential benefits of herbal remedies by providing a sufficient scientific basis for their mechanism of action, any possible side effects, and any contraindications, as well as assessing their concerning safety aspect [3, 4]. In particular, the study of the immunomodulatory effects of plants has gained significant attention, as the immune system plays a crucial role in maintaining health and preventing disease, especially during the recent pandemic outbreak. This particular global event also put immunology studies in the general public spotlight because it raised public concern about the consequences of contracting the disease, which could clinically lead to severe morbidity and mortality [5]. The general population with a controlled immune system frequently did not contract the disease because their immune system worked adequately to protect healthy cells and immediately neutralized or eliminated infectious agents, preventing further disease progression [6]. However, small subsets of the population with immunocompromised conditions, such as seniors, organ transplant recipients, chemotherapy patients, and AIDS patients, as well as a wide range of autoimmune-bearing people, were at a higher risk of worsening disease prognosis, such as complete loss of immune tolerance, chronic inflammation, and even fatal hypercytokinemia [7–10].

Both innate and adaptive immune cells are controlled by the expression level of cytokines such as TGF-β, IL-2, IL-4, IL-6, IL-10, IL-33, TNF-α/β, and IFN-γ in a regular immune system to provide proper responses to infection, regulate inflammation, promote immunological memory, and crucially sustain immune homeostasis [11, 12], and specialized regulatory cells with their respective cytokines, namely regulatory T cells (Tregs), B cells, macrophages, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC) [13–15]. Tregs play a crucial role in regulating immune responses and preventing autoimmune diseases. Bregs, on the other hand, help regulate immune responses by producing cytokines that suppress inflammation, including IL-10 and IL-35. Mregs and DCs are involved in the resolution of inflammation, while MDSCs play a role in suppressing immune responses and promoting tolerance. Their interaction with every involved immune cell will fundamentally manipulate the expression of other cytokines in an interconnected manner. Tregs and Bregs, for example, can suppress the production of pro-inflammatory cytokines like TNF-α and IL-6, preventing excessive inflammation [16, 17].

Additionally, external factors, especially diet, can strongly influence those complex positive and negative pathways [18, 19]. Dietary intake of vegetables, fruits, herbs, and spices through foods or even herbal medicine could support immune homeostasis because they contain micronutrients such as vitamins and various natural bioactive compounds, which could directly or indirectly influence immune homeostasis. Herbal medication, on the other hand, is often derived from endemic plants, which are plants that are native to specific regions and are less well-known outside of those regions. Despite this, these plants have been used for centuries in traditional medicine to treat various conditions, including those related to the immune system [20–21]. One example is the ant-plant, also known as baboon's head, which belongs to the Rubiaceae family. This epiphytic plant has a noteworthy mutualistic relationship with an ant colony that allows it to survive. It possesses structural adaptations that provide ants with food and shelter, distinguishing it due to this unusual interdependence with its ant symbionts. The two most studied species are *Myrmecodia pendans* and *Myrmecodia tuberosa,* which are thought to have therapeutic potential. Some indigenous tribes in New Guinea have long used this plant as a medication to cure various chronic conditions such as headaches, inflammation, tumors, renal problems, and rheumatism [22, 23]. However, this study used the less-common and less-endangered species from the *Hydnophytum* genus called *Hydnophytum formicarum*. It has the same common name in Southeast Asia as its *Myrmecodia* counterparts but is less scientifically studied.

Among the various bioactive compounds predicted to be present in *H. formicarum*, several major bioactive compounds are expected to be present, including β-sitosterol, stigmasterol, 4-aminophenyl acetate, isoliquiritigenin, butin, butein, proto-catechualdehyde, and aucubin [24, 25]. Most of them are classified as potent immunomodulators because in controlled studies, they demonstrate the ability to modulate the immune system in a significant and observable way by affecting the activation, proliferation, and function of immune cells, mainly to restore immune imbalance [26]. In this study, we focused on the lymphocytes, particularly $CD4^+$, $CD8^+$, $CD4^+CD25^+$, and $B220^+$ cells, by employing a mouse model to examine the quantitative lymphocyte changes after extract injection. Furthermore, we also study the ideal dose of the *H. formicarum* extracts as therapeutic, identified by the histopathology of the liver because it is a critical organ for drug metabolism and excretion. Moreover, drug-induced liver injury is a common adverse effect of many drugs, and the severity of this injury can be assessed through histopathological examination of liver tissue [27–29].

Materials and Methods

Preparation of *H. formicarum* Tuber Extract

The ant-plant tuber powder was prepared from four kg of *H. formicarum* tuber. After being cleaned, the tuber was peeled, chopped, and sundried for 3–5 days. After that, it was dried again in an oven at 60^{o}C. The dried tuber was then powdered using a blender and refined using a sieve to achieve uniform consistency. The powdered ant-plant tuber was dissolved in hot water to prepare a stock solution. The filtered extract was then orally administered to the experimental mouse model.

Experimental Animals and Treatments Description

The BALB/c mice (*Mus musculus*) used in this study were six weeks old and in good health, with active locomotion, no hair loss, and legs that were not bent or malformed. This study employed twelve mice divided into four treatment groups: no treatment group, 100 mg/kg of BW of tuber extract (HFE100 group), 500 mg/kg of BW (HFE500 group), and 1000 mg/kg of BW (HFE1000 group), with a total of three replications for each. Most of the experiment was conducted at Department of Biology, Brawijaya University for approximately six months. Importantly, this experiment had been reviewed by the Ethics Committee of Brawijaya University with ethical clearance No. 92-KEP-UB.

Lymphoid Organs Isolation

The lymphoid organs, particularly the spleen and bone marrow, were isolated. The mice were sacrificed by neck dislocation and dissected to obtain the spleen. The spleen was then washed thrice in sterile PBS, put on a petri dish filled with sterile PBS, and pressed with the syringe's base clockwise from top to bottom. Furthermore, the crushed spleen is filtered with wire and placed in a propylene tube. The propylene tube suspension was mixed with 8 ml of PBS and centrifuged at 1500 rpm for five minutes at 15°C, then the supernatant was discarded, and the pellet was suspended and homogenized in 1 ml of PBS. The suspended pellet was then transferred to a micro tube with 1 mL of PBS, then centrifuged again for three minutes at 2500 rpm at 15°C. It was then kept at 4°C for quantitative examination with flow cytometry.

Meanwhile, the femur was cleaned of any remaining tissue and muscles from the dissected mice for bone marrow isolation. Then, using a 1 mL syringe, spray the base of the bone with PBS. The sample was then aspirated using a micropipette and filtered with wire. After that, pour it into an 8 ml tube. Furthermore, the bone marrow suspension was processed in the same procedure as the spleen before the flow cytometry analysis stage.

Flow Cytometry Analysis of $CD4^{+}$, $CD8^{+}$, $CD4^{+}CD25^{+}$ and $B220^{+}$ Cells

The pellets in the microtube were then treated with anti-CD4 antibodies, anti-CD8 antibodies, anti-CD4 antibodies, anti-CD25 antibodies, and anti-B220/CD45 antibodies before

being kept on ice. After that, the antibody-treated pellet was pipetted into a cuvette tube on a flow cytometer with a micropipette, and 400 microliters of sterile PBS were added and homogenized by pipetting. The cuvette is pushed against the nozzle of the BD Biosciences FASCalibur flow cytometer for quantitative analysis according to our previous study [19,20].

Liver Slide Preparations and Histopathological Observations

The liver was isolated, particularly the middle lobe. The samples were cleaned three times with sterile PBS before being placed in a 4% PFA fixative solution diluted with PBS for 1–7 days. The samples were dehydrated in a 70% ethanol series for 24 hours, in 80%, 90%, and 95% ethanol for 30 minutes each, then immersed in 100% ethanol three times for 30 minutes each. The samples were then submerged in xylol for 30 minutes at room temperature, followed by 30 minutes in another xylol solution. The samples were then immersed in xylol: paraffin mixture sequentially with the ratio of 3:1, 1:1, and 1:3 and pure paraffin for 30 minutes at temperatures ranging from 56 to 58^{o}C. After being dipped in liquid paraffin, the samples were embedded in a block mold. The air bubbles were removed from the paraffin liquid and held at 40°C until the paraffin solidified. A microtome was used to slice the organs to 5 μm. With a brush, the cut ribbon was placed in warm water on a metal tray placed on a hot plate set at 40^{o}C. The perfectly stretched slices were taken using an object glass. Furthermore, the paraffin tape was cured, adhered to the object glass, and put on a hot plate (38–40°C).

The samples were deparaffinized by soaking them in xylol twice for 10 minutes. The slides were then submerged in graded ethanol (100%, 95%, 90%, 80 %, 70%, 60%, and 30%) for 5 minutes. After washing the slides with distilled water for five minutes, they were stained with hematoxylin for ten seconds. The preparations were rinsed with distilled water for 5 minutes before submerging in 30%, 60%, and 70% graded ethanol for 5 minutes each. The slides were then stained with eosin for 1 minute each, for 5 minutes, then cleaned with xylol three times for five minutes each before being mounted. Hematoxylin dye will turn the cell nucleus purple, whereas Eosin dye will turn the cytoplasm red. The slides were histopathologically examined using a BX51 digital imaging microscope.

Data Analysis

This research employed a one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test to investigate the relative abundance of the subset of immune cells (Figure 1). The p-value <0.05 indicates the significance between group. All of the statistical analysis was carried out by SPSS 26.0 for Windows.

Results and Discussion

Total $CD4^+$ and $CD8^+$ Cell Number Analysis in Spleen

The flow cytometry results showed that the $CD4^+$ cell number in the spleen fluctuates along with the treatment's dosage. The dose I and III treatments showed lower numbers of $CD4^+$ cells, with 15.60% and 17.24%, respectively, compared to the control (17.53%), while dose II showed a higher cell count of 19.34%. It showed no difference with the $CD8^+$ numbers, either. Doses I and II had lower numbers, with 10.49% and 8.60%, compared to the control (10.55%), while dose III increased to 12.14%. When we calculated the $CD4^+/CD8^+$ ratio, we got 1.487, 2.248, 1.420, and 1.661 for doses I, II, III, and the control group, respectively (Figure 2). Although the ratio remained higher than the consensus value of 1.0, the ratio for the treatment groups tended to drop compared to the control, except for the dose II treatment. Despite the fluctuating number, those results are statistically insignificant ($p > 0.05$). Among the treatment groups, the lowest dose consistently suppressed the number of T cells, while the two others increased one type while decreasing the other.

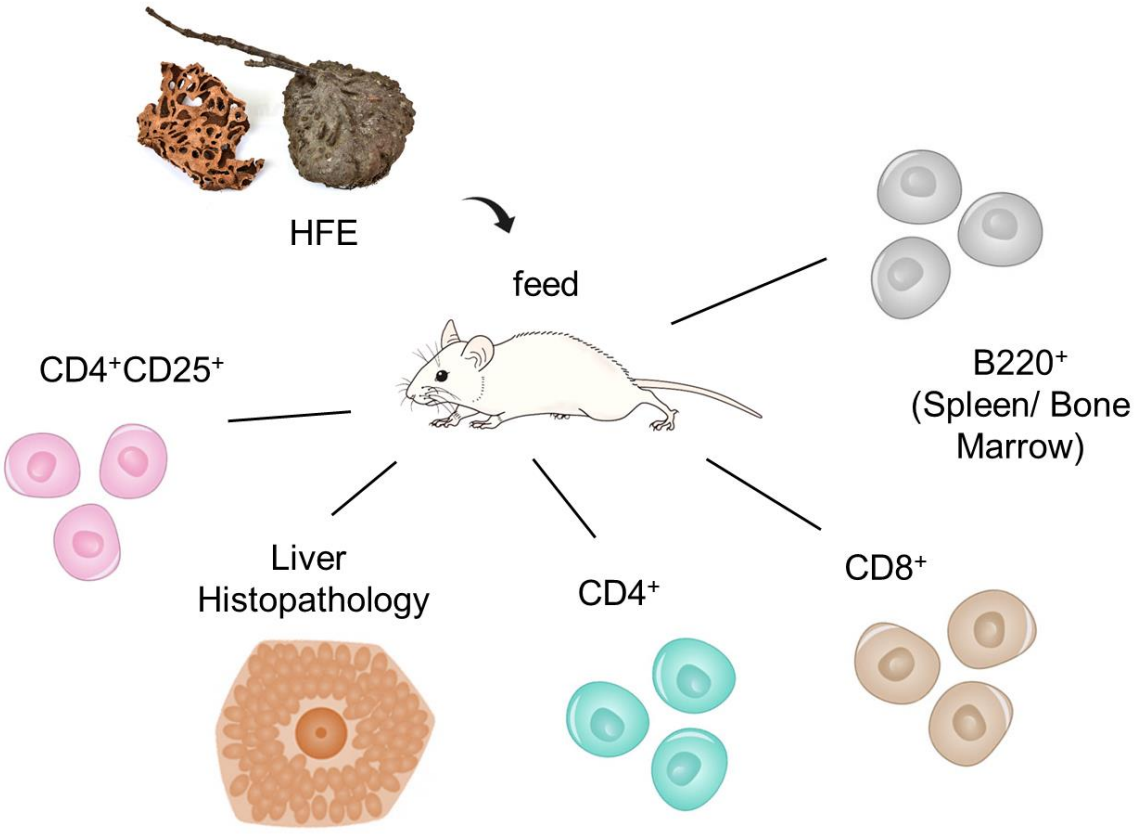

Figure 1:- Several immune cell subsets and histopathological assessments were evaluated following HFE treatment.

Both $CD4^+$ and $CD8^+$ numbers showed a similar trend. They fluctuated, but not in a dose-dependent way. The dose I supplements suppresses both T cell subtypes, whereas dose III supplements increase the number of $CD8^+$ cells while decreasing the number of $CD4^+$ cells. The only ideal treatment results shown in the dose II group were an increasing $CD4^+$ number and a decreasing $CD8^+$ number, the exact opposite of the dose III results, which also had a 100% higher dose.

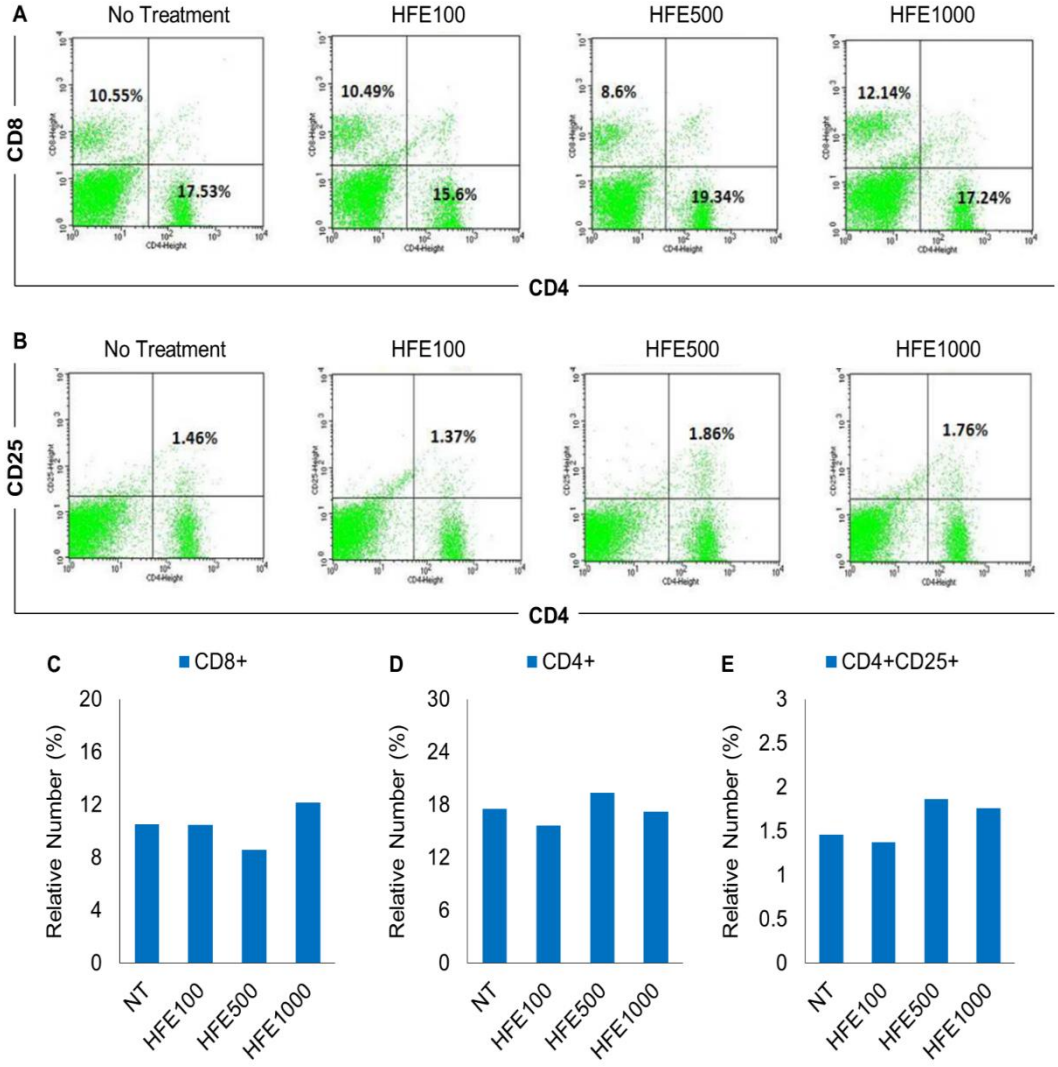

Figure 2- Evaluating of immunomodulation activity of HFE on CD8, CD4, and CD4CD25 T cells (A). Flow cytometry graph of $CD8^+/CD4^+$; (B). Flow cytometry graph of $CD4^+CD25^+$; (C). Bar graph of $CD8^+$; (D). Bar graph of $CD4^+$; and (E). Bar graph of $CD4^+CD25^+$.

In the extract, we suspect that major bioactive compounds with similar molecular structure, β-sitosterol and stigmasterol, could influence the number of both T cell subsets. Most studies on the effect of this compound on immune cells suggest that it promotes the expression of anti-inflammatory cytokines like IL-10, which then suppresses pro-inflammatory cytokines like IL-

1β and TNF-α via inhibition of the NF-κB/JAK pathway, particularly by downregulating NF-κB itself [30, 31]. Besides, aucubin and isoliquiritigenin also exert a similar effect, with the latter inhibiting IL-6 and IL-8 expression [32]. The inhibition of NF-κB activity could also hamper the expression of various genes involved in the pro-inflammatory response, including IL-2, which contributes to T cell expansion and differentiation by lowering the PI3K/Akt/mTOR pathway activation. This exact mechanism is also thought to be induced by butein, another primary bioactive compound in *H. formicarum* [33, 34]. These mechanisms could hamper the number of T cells, though this seems insignificant in this study.

The effect of β-sitosterol on both T cell subset numbers could be attributed to increased IL-12, IL-18, and IFN-γ, which later increased the population of $CD4^+$ and $CD8^+$ T cells, according to a study using a colon carcinoma mouse model [35]. These explanations only aligned with the minority of the results and directly opposed the majority. Although they did not follow a dose-dependent pattern, the majority of the results showed that the number of $CD4^+$ and $CD8^+$ cells is significantly lower than the control, which could be due to the interaction between β-sitosterol and, later, stigmasterol and the T cell receptor and its corresponding downstream pathways [31]. These contradictory findings and explanations call for more research into the precise mechanism by which β-sitosterol influences T cell number and cytokine expression. The *H. formicarum* extract also contains significant amounts of other compounds, including butin. Although it has not been specifically studied, it has been shown to reduce the level of mitochondrial malfunction brought on by oxidative stress [36], which theorizes should increase the quantity of both $CD4^+$ and $CD8^+$ T cells.

Another interesting point is how the ratio of $CD4^+/CD8^+$ shifted among the treatment groups. All treatment groups other than dose II had worse ratios than the control, although they did not fall below the common consensus value for healthy individuals at 1.0 [37, 38]. It also did not follow any meaningful pattern because the values seemed to fluctuate and did not correlate with the experimental dose. From this perspective, we could assume that the lowest dose is too low to improve the state, while the highest dose seems to promote the number of $CD8^+$ T cells over $CD4^+$ T cells. Meanwhile, the second dose may promote a balance between the two subsets. Another possibility is that the lowest and highest doses are causing toxicity to the T cells, leading to a decrease in their numbers and a decrease in the $CD4^+/CD8^+$ ratio. Toxicity can be caused by various mechanisms, such as cell death or inhibition of cell proliferation, and can be the result of the treatment's direct effects on the cells or indirect effects on other cell types or molecules in the immune system. The second dose might represent an optimal balance, where the treatment continues to foster T-cell proliferation or survival without inducing toxicity. The characteristics of bioactive compounds, particularly flavonoids known for their dual nature as immunostimulants and immunosuppressive agents, could potentially explain the

inconsistencies observed in the results. At a particular dosage level, cytotoxic and immunosuppressive effects allow for suppressing lymphocyte growth. It might explain why there is no discernible change in the number of T cells between treatments and controls.

Total $CD4^+CD25^+$ Cell Number Analysis in Spleen

The subset of T cells that bind self-antigens with high avidity but do not reach the elimination stage was developed into regulatory T cells (Treg), which are critical for inhibiting the autoreactivity of cells that pass negative selection [39, 40]. These subsets of T cells also hold a vital role in preventing the onset of autoimmune diseases. Treg cells are a subset of $CD4^+$ T cells that express the IL-2Rα (CD25) chain and are stationed in lymphoid tissue and inflammatory sites [40, 41]. Treg cells produce the immunosuppressive cytokine IL-10, which suppresses T cell and macrophage proliferation, as well as IFN-γ activity produced by activated macrophages. They also synthesize TGF-β, inhibiting T and B cell proliferation [42]. The flow cytometry analysis on spleen cells reveals that the number of $CD4^+CD25^+$ cells varies between treatment groups. The relative cell at the dose I decreased to 1.37%, compared to the control of 1.46%. Meanwhile, the rest of the doses increased by 1.86% and 1.76% for the dose II and III groups, respectively (Figure 2). However, it is worth noting that all the results are statistically insignificant.

One explanation for the low number of regulatory T cells (Tregs), also known as $CD4^+CD25^+$ T cells, is that the mouse models were not exposed to any infection or antigen. We believe the Treg population detected is classified as naturally occurring Treg cells because the study's design did not expose the mouse model to any antigens. We noticed that the number is consistently lower than 2%, which is considered far below the normal range of around 5–10% [43, 44]. However, other studies suggest a broader normal range with a lower limit of as low as 0.6% and an upper limit of as high as 10%, which makes the results completely within the normal range [45, 46]. We assumed that, based on the negative control group as a baseline, we could neglect those range problems. This aspect becomes a serious matter because any abnormal number of Tregs could hamper the immune homeostasis through progressing autoimmune diseases, immunodeficiency, or as a marker for inflammation and progressing disease, particularly cancer [47, 48]. Meanwhile, according to the same study, a low population of $CD4^+CD25^+$ T cells is not the only indicator of a low Treg population because FoxP3 expression is a more prominent and definitive marker for Tregs. There is also a population of Tregs that are marked as $CD4^+FoxP3^+$ with low or completely absent CD25 expression, which makes them undetectable with our combination of antibodies, which only incorporate anti-CD4 and anti-CD25 without anti-FoxP3. This phenomenon may indicate a greater shift in Tregs from a naive-like CD25hi phenotype to a CD25-/low memory, or effector, phenotype [48]. Hence, we cannot conclusively determine if the results follow that mechanism or not because

we did not incorporate the FoxP3 marker in this study.

Except for the dose I group, all other treatment groups have a higher number of Tregs than the control, but not in a dose-dependent manner. As mentioned in the previous section, the consequence of having the AKT/mTOR pathway suppressed and/or AMPK activated by a bioactive compound such as butin and isoliquiritigenin is that it induces fatty acid oxidation as a primary source of energy, which subsequently drives the activated T cells' phenotype into Tregs [34, 49–51]. Other compounds, namely β-sitosterol and stigmasterol, could also restore the Th17/Treg balance in an inflammatory bowel disease case [52, 53]. We do not have a clear explanation for this phenomenon locally in the spleen. However, it is thought to be related to the activation of the peroxisome proliferator-activated receptor-γ (PPARγ) pathway, which is critical for fatty acid oxidation and thus promoting Treg differentiation [24]. Butein, on the other hand, is predicted to influence the number of Treg cells with a different approach. Based on its capacity as an antioxidant, it suppresses the number of iNOS on $CD4^+$ T cells, which then promotes Treg induction [54]. Butein, on the other hand, is predicted to influence the number of Treg cells with a different approach. Based on its antioxidant capacity, it suppresses the number of iNOS on $CD4^+$ T cells, promoting Treg induction [55–57]. We also found that aucubin, protocatechualdehyde, and butein inhibit NF-κB activity. Although it is still unclear and needs deeper understanding, we suspect this phenomenon could possibly force the activation of the non-canonical NF-κB pathway through its NF-κB2 subunit, whose corresponding downstream activation would subsequently increase the number of Tregs [58–60]. Also, it is worth noting that the results are statistically insignificant and inconsistent, so further evaluation is critically needed.

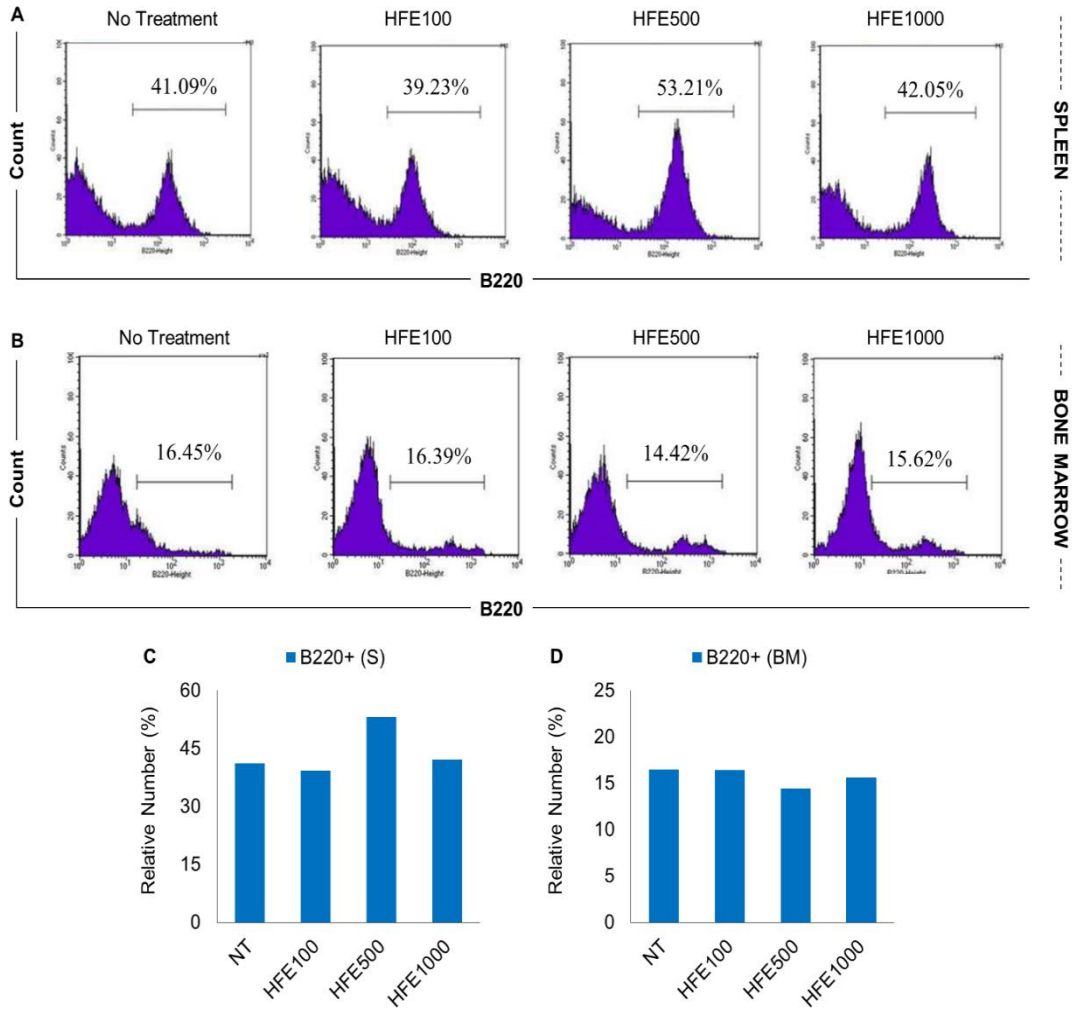

Figure 3. Evaluating of immunomodulation activity of HFE on B220 cells in spleen and bone marrow (A). Flow cytometry graph of $B220^+$ in the spleen; (B). Flow cytometry graph of $B220^+$ in the bone marrow; (C). Bar graph of $B220^+$ in spleen; and (D). Bar graph of $B220^+$ in the bone marrow.

Total $B220^+$ Cell Number Analysis in the Spleen and Bone Marrow

The class of cells that can produce antibodies is known as B cells. They make up the vast majority of the humoral adaptive immune system. Their major objective is to produce specific antibodies that prevent or quickly neutralize infectious agents, particularly eliminating the extracellular pathogens, after being activated through certain activation pathways, mainly driven by the identification of the foreign antigen [40]. The B cells express the B220 (CD45) surface antigen, so they are often notified as $B220^+$ cells. The B220 is an isoform of CD45 antigen, commonly called pan B cell marker and cell differentiation antigen in mice. In humans, it was expressed only by a subset of B cells that did not express CD27, a marker for memory B cells [40, 61].

The population count of $B220^+$ expressing cells in bone marrow showed a decreasing trend,

yet it was statistically insignificant compared to the control group, but it did not appear to be dose-dependent. The results for doses I, II, and III were 16.39%, 14.42%, and 15.62%, respectively, lower than the control at 16.45%. Compared to its counterpart in the bone marrow, the cell number in the spleen is more fluctuated, with no single decreasing or increasing trend, and it also does not follow a dose-dependent pattern. Except for dose I, with a value of 39.23%, the rest of the treatment groups showed an increased number compared to the control, with the values of doses II, III, and control at 53.21, 42.05, and 41.09%, respectively. Contrary to previous results in the bone marrow, these results are statistically significant. Furthermore, when the interaction between organs and dosage is considered, there is no considerable influence on the relative number of $B220^+$ cells (Figure 3).

Among the treatment dosages, the dose I group showed persistent suppression activity towards $B220^+$ cells in the bone marrow and spleen. Unfortunately, we cannot find any scientifically possible explanation for the phenomenon. One of the most well-known reasons is that several bioactive compounds such as aucubin, isoliquiritigenin, protocatechualdehyde, and butein block the activation of NF-κB, which subsequently decreases the expression of pro-inflammatory cytokines decreases, as does the capacity of T cells to influence B cell activity [62]. The observed reduction in the number of B220+ cells is proposed to be primarily attributed to the highest dosage. In contrast, the other two dosages consistently diminished the count of B220+ cells in the bone marrow while simultaneously exhibiting an opposite effect, leading to an increase in the number of B220+ cells in the spleen. The increased number of B cells in the spleen could be attributed to the extract's β-sitosterol and stigmasterol content, which stimulate IL-10 expression and thus increase B cell survival and proliferation [63–65]. This phenomenon may be related to the fluctuating numbers of $CD4^+$ T cells and $CD4^+CD25^+$ Tregs discussed in the previous section. The splenic B cells, $CD4^+$ T cells, and $CD4^+CD25^+$ Tregs showed similar patterns. We suspect the Th2 subset, the primary source for IL-10, could dominate an increased CD4+ T cell subset. Besides, it also aligns with the $CD4^+CD25^+$Tregs number, also the primary source of IL-10 [66]. However, a newer study suggests that β-sitosterol administration could result in lower serum concentrations of IL-10 and IL-1β and IL-6 in mouse sepsis models [67]. Despite all negative attribution, reactive oxygen species (ROS) are also key components in regulating B cells, including their proliferation. However, it only functions as a regulator when the dose is controlled [68]. On the other hand, increased oxidants in B cells also could cause spontaneous apoptosis [69]. Antioxidant properties of various bioactive compounds could alleviate and balance the oxidative stress caused by ROS in B cells, which could extend its lifespan and prevent it from undergoing premature apoptosis.

Liver Toxicity Analysis after HFE Administration

The liver is one of the organs most vulnerable to injury from drug-induced liver injury (DILI), a common adverse effect of all medications, including medicinal herbs, which may eventually result in liver failure [70]. The most prominent manifestations of DILI are liver necrosis, apoptosis, and necroptosis, depending on the activated pathway due to the induction of organelle stress, which leads to hepatocyte death [71]. The hepatocyte abnormalities could be applied to assess liver tissue injury. Furthermore, the presence of sinusoids and veins can be used to detect anomalies in liver histology [72–74]. The hepatic sinusoids are convoluted and dilated blood veins with uneven diameters lined with incompletely stratified endothelial cells and isolated from the underlying hepatocytes by the perisinusoidal gap [75, 76]. Based on the histopathological slide of a mouse's liver, we determined the toxicity level of *H. formicarum* crude extract from the experimental doses. Based on the histological image, it is clear that there is no substantial difference between the treatments (Figure 4). However, a form of degeneration with a low percentage was found. It was also insignificant among the dosages and the control, implying that all doses did not harm hepatocytes or trigger necrosis. Following our investigation, no evidence was found indicating that administration of H. formicarum induced liver injury in the form of apoptosis or necrosis in similar studies. Another study suggests that administering an aqueous extract of the ant-plant from different species, namely *M. pendans* is safe enough to be developed as a therapeutic raw material because there is no LD50 value and no mortality even at dosages up to 3750 mg/kg of BW [77]. We assumed that both ant-plant species possessed similar degrees of hepatotoxicity.

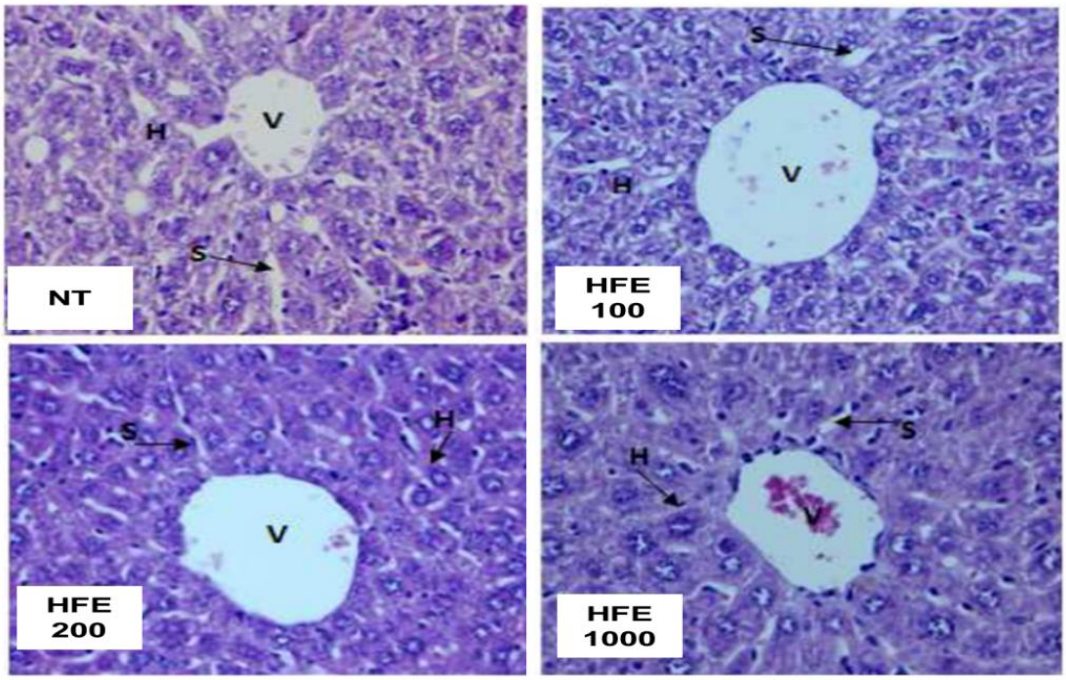

Figure 4: Microphotograph of hepatocytes section from experimental mice model under HFE treatment (HE stains, M = 400×).

Due to their "natural" origin, it is a prevalent misconception that herbal drugs cause less side effects than their commercial counterparts. However, it is not entirely risk-free and foolproof due to the immense variety of chemical ingredients and unknown individual dosages. Increased serum levels of the two most well-known markers of hepatocyte injury, serum glutamate oxaloacetate transferase (SGOT) and serum glutamate pyruvate transferase (SGPT) imply that it was harmful to hepatocytes in experiments utilizing a different tuber from *T. flagelliforme* [78, 79]. These findings highlight the need for caution when using herbal drugs and the importance of conducting thorough studies to assess their safety.

Conclusion

According to the findings, the immune cell subsets including $CD8^+$, $CD4^+$, $CD4^+CD25^+$, and $B220^+$ cells had been affected by *H. formicarum* in a few spots. It is important to note that the histopathological view demonstrated that the control and treatment groups do not significantly different. Therefore, the treatment of *H. formicarum* might not influence the liver in the negative way. Importantly, future research related to the other immunological aspects and the toxicity of the *H. formicarum* is necessary.

Acknowledgment

The authors thank Brawijaya University for providing a research grant (M.R.) and supporting this study.

Conflict of Interest

No conflict of interest

References

[1] C. S. J. Woo, J. S. H. Lau, and H. El-Nezami, "Chapter 10 - Herbal Medicine: Toxicity and Recent Trends in Assessing Their Potential Toxic Effects", in *Advances in Botanical Research*, L.-F. Shyur and A. S. Y. Lau, Eds., in Recent Trends in Medicinal Plants Research, vol. 62. Academic Press, 2012, pp. 365–384.

[2] H. Yuan, Q. Ma, L. Ye, and G. Piao, "The Traditional Medicine and Modern Medicine from Natural Products", *Molecules*, vol. 21, no. 5, pp. 1–18, 2016.

[3] M. Ekor, "The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety", *Frontiers in Pharmacology*, vol. 4, pp. 1–10, 2014.

[4] X. Zhou, C.-G. Li, D. Chang, and A. Bensoussan, "Current status and major challenges to the safety and efficacy presented by Chinese herbal medicine", *Medicines*, vol. 6, no. 1, pp. 1–10, 2019.

[5] L. Han, Q. Fu, C. Deng, L. Luo, T. Xiang, and H. Zhao, "Immunomodulatory potential of flavonoids for the treatment of autoimmune diseases and tumour", *Scandinavian Journal of Immunology*, vol. 95, no. 1, pp. 1–19, 2022.

[6] N. D. Huntington and D. H. Gray, "Immune homeostasis in health and disease", *Immunology & Cell Biology*, vol. 96, no. 5, pp. 451–452, 2018.

[7] C. D. Bertini, F. Khawaja, and A. Sheshadri, "COVID-19 in the Immunocompromised Host", *Clinics in Chest Medicine*, 2022.

[8] R. Murtas, A. Andreano, and F. Gervasi, "Association between autoimmune diseases and COVID-19 as assessed in both a test-negative case–control and population case–control design", *Autoimmunity Highlights*, vol. 11, no. 1, pp. 1–10, 2020.

[9] L. Amaya-Uribe, M. Rojas, G. Azizi, J.-M. Anaya, and M. E. Gershwin, "Primary immunodeficiency and autoimmunity: A comprehensive review", *Journal of Autoimmunity*, vol. 99, pp. 52–72, 2019.

[10] E. Monreal, S. Maza, and F. Velasco, "The Impact of Immunosuppression and Autoimmune Disease on Severe Outcomes in Patients Hospitalized with COVID-19", *Journal of Clinical Immunology*, vol. 41, no. 2, pp. 315–323, 2021.

[11] I. S. Pires, P. T. Hammond, and D. J. Irvine, "Engineering Strategies for Immunomodulatory Cytokine Therapies: Challenges and Clinical Progress", *Advanced Therapeutics*, vol. 4, no. 8, pp. 1–29, 2021.

[12] A. Kelly, S. A. Houston, E. Sherwood, J. Casulli, and M. A. Travis, "Chapter Four - Regulation of Innate and Adaptive Immunity by TGFβ", in *Advances in Immunology*, F. W. Alt, Ed., Academic Press, 2017, pp. 137–233.

[13] T. Cao, S. Shao, H. Fang, B. Li, and G. Wang, "Role of Regulatory Immune Cells and Molecules in Autoimmune Bullous Dermatoses", *Frontiers in Immunology*, vol. 10, pp. 1–11, 2019.

[14] S. Saha, I. N. Shalova, and S. K. Biswas, "Metabolic regulation of macrophage phenotype and function", *Immunological Reviews*, vol. 280, no. 1, pp. 102–111, 2017.

[15] S. Ness, S. Lin, and J. R. Gordon, "Regulatory Dendritic Cells, T Cell Tolerance, and Dendritic Cell Therapy for Immunologic Disease", *Frontiers in Immunology*, vol. 12, pp. 1–18, 2021.

[16] F. N. Mohd Jaya, S. G. Garcia, F. E. Borràs, G. C. F. Chan, and M. Franquesa, "Paradoxical role of Breg-inducing cytokines in autoimmune diseases", *Journal of Translational Autoimmunity*, vol. 2, pp. 1–12, 2019.

[17] H. Zhao, L. Wu, and G. Yan, "Inflammation and tumor progression: signaling pathways and targeted intervention", *Signal Transduction and Targeted Therapy*, vol. 6, no. 1, 2021.

[18] W. E. Putra, W. O. Salma, and M Rifa'i, "Anti-inflammatory Activity of Sambucus Plant Bioactive Compounds against TNF-α and TRAIL as Solution to Overcome Inflammation Associated Diseases: The Insight from Bioinformatics Study", *Natural Product Sciences,* vol. 25, no. 3, pp. 215-221, 2019.

[19] W. E. Putra, "In silico study demonstrates multiple bioactive compounds of Sambucus plant promote death cell signaling pathway via Fas receptor", *FUW Trends in Science & Technology Journal*, vol. 3, no. 2, pp. 682-685. 2018.

[20] W. E. Putra, and M. Rifa'i, "Immunomodulatory Activities of *Sambucus javanica* Extracts in

DMBA Exposed BALB/c Mouse", *Advanced Pharmaceutical Bulletin*, vol. 9, no. 4, pp. 619-623, 2019.

[21] W. E. Putra, and M. Rifa'i, "Hematopoiesis Activity of *Sambucus javanica* on Chloramphenicol-induced Aplastic Anemia Mouse Model", *Natural Product Sciences*, vol. 25, no. 1, pp. 59-63, 2019.

[22] T. Hertiani, E. Sasmito, Sumardi, and M. Ulfah, "Preliminary Study on Immunomodulatory Effect of Sarang-Semut Tubers *Myrmecodia tuberosa* and *Myrmecodia pendens*", *OnLine Journal of Biological Sciences*, vol. 10, no. 3, pp. 136–141, 2010.

[23] A. S. Nugraha, B. Triatmoko, P. Wangchuk, and P. A. Keller, "Vascular Epiphytic Medicinal Plants as Sources of Therapeutic Agents: Their Ethnopharmacological Uses, Chemical Composition, and Biological Activities", *Biomolecules*, vol. 10, no. 2, 2020.

[24] A. S. Nugraha, T. Wangchuk, A. C. Willis, R. Haritakun, H. Sujadmiko, and P. A. Keller, "Phytochemical and Pharmacological Studies on Four Indonesian Epiphytic Medicinal Plants: Drynaria rigidula, Hydnophytum formicarum, Usnea misaminensis, and Calymperes schmidtii", *Natural Product Communications*, vol. 14, no. 6, pp. 1–6, 2019.

[25] F. El Babili, G. Rey-Rigaud, H. Rozon, and B. Halova-Lajoie, "State of knowledge: Histolocalisation in phytochemical study of medicinal plants", *Fitoterapia*, vol. 150, pp. 1–12, 2021.

[26] I. Jantan, Md. A. Haque, M. Ilangkovan, and L. Arshad, "An Insight Into the Modulatory Effects and Mechanisms of Action of Phyllanthus Species and Their Bioactive Metabolites on the Immune System", *Frontiers in Pharmacology*, vol. 10, pp. 1–19, 2019.

[27] W. E. Putra, A. Soewondo, and M. Rifa'i. "Expression of erythroid progenitor cells and erythrocytes on dexamethasone induced-mice", *Biotropika*, vol. 3, pp. 42-45, 2015.

[28] W. E. Putra, and M. Rifa'i, "Assessing the Immunomodulatory activity of ethanol extract of *Sambucus javanica* berries and leaves in chloramphenicol-induced aplastic anemia mouse model", *Tropical Life Sciences Research*, vol. 31, pp. 175-185, 2020.

[29] D. E. Kleiner, P. C. Naga, and W. M. Lee, "Hepatic Histological Findings in Suspected Drug-Induced Liver Injury: Systematic Evaluation and Clinical Associations", *Hepatology*, vol. 59, no. 2, pp. 661–670, 2014.

[30] M. S. Valerio, H. Minderman, T. Mace, and A. B. Awad, "β-Sitosterol modulates TLR4 receptor expression and intracellular MyD88-dependent pathway activation in J774A.1 murine macrophages", *Cellular Immunology*, vol. 285, no. 1, pp. 76–83, 2013.

[31] C.-F. Le, T. H. Kailaivasan, S.-C. Chow, Z. Abdullah, S.-K. Ling, and C.-M. Fang, "Phytosterols isolated from Clinacanthus nutans induce immunosuppressive activity in murine cells", *International Immunopharmacology*, vol. 44, pp. 203–210, 2017.

[32] Y. Wu, X. Chen, and X. Ge, "Isoliquiritigenin prevents the progression of psoriasis-like symptoms by inhibiting NF-κB and proinflammatory cytokines", *Journal of Molecular Medicine,* vol. 94, no. 2, pp. 195–206, 2016.

[33] X. Bai, Y. Ma, and G. Zhang, "Butein suppresses cervical cancer growth through the PI3K/AKT/mTOR pathway", *Oncology Reports*, vol. 33, no. 6, pp. 3085–3092, 2015.

[34] A. Hosseinzade, O. Sadeghi, A. Naghdipour Biregani, S. Soukhtehzari, G. S. Brandt, and A. Esmaillzadeh, "Immunomodulatory Effects of Flavonoids: Possible Induction of T CD4+ Regulatory Cells Through Suppression of mTOR Pathway Signaling Activity", *Frontiers in Immunology,* vol. 10, pp. 1–12, 2019.

[35] C.-Y. Shen, C.-F. Lee, W.-T. Chou, J.-J. Hwang, Y.-S. Tyan, and H.-Y. Chuang, "Liposomal β-Sitosterol Suppresses Metastasis of CT26/luc Colon Carcinoma via Inhibition of MMP-9 and Evoke of Immune System", *Pharmaceutics*, vol. 14, no. 6, Art. no. 6, 2022.

[36] R. Zhang, K. Kang, and M. J. Pio, "Butin reduces oxidative stress-induced mitochondrial dysfunction via scavenging of reactive oxygen species", *Food and Chemical Toxicology*, vol. 48, no. 3, pp. 922–927, 2010.

[37] J. A. McBride and R. Striker, "Imbalance in the game of T cells: What can the CD4/CD8 T-cell ratio tell us about HIV and health?", *PLOS Pathogens*, vol. 13, no. 11, pp. 1–7, 2017.

[38] J. Tang, X. Li, and M. A. Price, "CD4:CD8 lymphocyte ratio as a quantitative measure of immunologic health in HIV-1 infection: findings from an African cohort with prospective data", *Frontiers in Microbiology*, vol. 6, pp. 1–9, 2015.

[39] K. G. Schmetterer, A. Neunkirchner, and W. F. Pickl, "Naturally occurring regulatory T cells: markers, mechanisms, and manipulation", *FASEB Journal,* vol. 26, no. 6, pp. 2253–2276, 2012.

[40] J. Owen, J. Punt, S. A. Stranford, and J. Kuby, *Kuby immunology*, 7. ed., International ed. Basingstoke: Macmillan Higher Education, 2013.

[41] P. P. Lui, I. Cho, and N. Ali, "Tissue regulatory T cells", *Immunology*, vol. 161, no. 1, pp. 4–17, 2020.

[42] A. K. Abbas, A. H. Lichtman, and S. Pillai, *Basic immunology: functions and disorders of the immune system*, Fifth edition. St. Louis, Missouri: Elsevier, 2016.

[43] E. V. Lourenço and A. La Cava, "Natural regulatory T cells in autoimmunity", *Autoimmunity*, vol. 44, no. 1, pp. 33–42, 2011.

[44] N. Ali and M. D. Rosenblum, "Regulatory T cells in skin", *Immunology*, vol. 152, no. 3, pp. 372–381, 2017.

[45] L. S. Taams, D. B. Palmer, A. N. Akbar, D. S. Robinson, Z. Brown, and C. M. Hawrylowicz, "Regulatory T cells in human disease and their potential for therapeutic manipulation", *Immunology*, vol. 118, no. 1, pp. 1–9, 2006.

[46] J. L. Pastrana, X. Sha, and A. Virtue, "Regulatory T cells and Atherosclerosis", *Journal of Clinical and Experimental Cardiology*, vol. 2012, no. Suppl 12, pp. 1–19, 2012.

[47] C. Li, P. Jiang, S. Wei, X. Xu, and J. Wang, "Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects", *Molecular Cancer*, vol. 19, no. 1, pp. 1–23, 2020.

[48] C. Dejaco, C. Duftner, B. Grubeck-Loebenstein, and M. Schirmer, "Imbalance of regulatory

T cells in human autoimmune diseases", *Immunology*, vol. 117, no. 3, pp. 289–300, 2006.

[49] I.-H. Sun, M. Oh, and L. Zhao, "mTORC1 signaling regulates the generation and function of central and effector FoxP3+ regulatory T cells", *Journal of Immunology,* vol. 201, no. 2, pp. 481–492, 2018.

[50] N. M. Chapman and H. Chi, "mTOR signaling, Tregs and immune modulation", *Immunotherapy*, vol. 6, no. 12, pp. 1295–1311, 2014.

[51] A. Guo, D. He, H.-B. Xu, C.-A. Geng, and J. Zhao, "Promotion of regulatory T cell induction by immunomodulatory herbal medicine licorice and its two constituents", *Scientific Reports*, vol. 5, no. 1, 2015.

[52] S. Wen, L. He, Z. Zhou, and R. Zhao. "Stigmasterol Restores the Balance of Treg/Th17 Cells by Activating the Butyrate-PPARγ Axis in Colitis", *Frontiers in Immunology*, vol. 12, pp. 1–17, 2021.

[53] Y.-Y. Tan, Y. Ding, X. Zheng, and G. Dai, "Ding's herbal enema treats dextran sulfate sodium-induced colitis in mice by regulating the gut microbiota and maintaining the Treg/Th17 cell balance", *Experimental and Therapeutic Medicine*, vol. 22, no. 6, pp. 1–11, 2021.

[54] S. Bakrim, N. Benkhaira, I. Bourais, and T. Benali, "Health Benefits and Pharmacological Properties of Stigmasterol", *Antioxidants*, vol. 11, no. 10, 2022.

[55] I. Navasardyan and B. Bonavida, “Regulation of T Cells in Cancer by Nitric Oxide”, *Cells*, vol. 10, no. 10, pp. 1–22, 2021.

[56] J. Sung and J. Lee, "Anti-Inflammatory Activity of Butein and Luteolin Through Suppression of NFκB Activation and Induction of Heme Oxygenase-1", *Journal of Medicinal Food*, vol. 18, no. 5, pp. 557–564, 2015.

[57] P. Jayaraman, M. Alfrano, and P. Svider, "iNOS expression in CD4+ T cells limits Treg induction by repressing TGFβ1: combined iNOS inhibition and Treg depletion unmask endogenous antitumor immunity", *Clinical Cancer Research*, vol. 20, no. 24, pp. 6439–6451, 2014.

[58] Y. Grinberg-Bleyer, R. Caron, and J. Seeley, "The alternative NF-κB pathway in regulatory T cell homeostasis and suppressive function", *Journal of Immunology*, vol. 200, no. 7, pp. 2362–2371, 2018.

[59] S.-N. Wang, G. Xie, and C. Qin, "Aucubin prevents interleukin-1 beta induced inflammation and cartilage matrix degradation via inhibition of NF-κB signaling pathway in rat articular chondrocytes", *International Immunopharmacology*, vol. 24, no. 2, pp. 408–415, 2015.

[60] N. Wang, W. Chen, and C. Cui, "The Peanut Skin Procyanidins Attenuate DSS-Induced Ulcerative Colitis in C57BL/6 Mice", *Antioxidants*, vol. 11, no. 11, 2022.

[61] R. Sj, S. A, L. B, and D. Dm, "The CD45 isoform B220 identifies select subsets of human B cells and B-cell lymphoproliferative disorders", *Human pathology*, vol. 36, no. 1, pp. 51–57, 2005.

[62] S. Kany, J. T. Vollrath, and B. Relja“, Cytokines in Inflammatory Disease,” *International Journal of Molecular Sciences*, vol. 20, no. 23, pp. 1–31, 2019.

[63] F. Jie, X. Yang, B. Yang, Y. Liu, L. Wu, and B. Lu", Stigmasterol attenuates inflammatory

response of microglia via NF-κB and NLRP3 signaling by AMPK activation," *Biomedicine & Pharmacotherapy*, vol. 153, pp. 1–11, 2022.

[64] M. Valerio and A. B. Awad, "β-Sitosterol down-regulates some pro-inflammatory signal transduction pathways by increasing the activity of tyrosine phosphatase SHP-1 in J774A.1 murine macrophages", *International Immunopharmacology*, vol. 11, no. 8, pp. 1012–1017, 2011.

[65] R. Liu, D. Hao, and W. Xu, "β-Sitosterol modulates macrophage polarization and attenuates rheumatoid inflammation in mice", *Pharmaceutical Biology*, vol. 57, no. 1, pp. 161–168, 2019.

[66] T. H. S. Ng, G. Britton, E. Hill, J. Verhagen, B. Burton, and D. Wraith, "Regulation of Adaptive Immunity; The Role of Interleukin-10", *Frontiers in Immunology*, vol. 4, pp. 1–13, 2013.

[67] S. Kasirzadeh, M. Ghahremani, and N. Setayesh, "β-Sitosterol Alters the Inflammatory Response in CLP Rat Model of Sepsis by Modulation of NFκB Signaling", *BioMed Research International*, vol. 2021, p. 5535562, 2021.

[68] E. Y. Bassoy, M. Walch, and D. Martinvalet, "Reactive Oxygen Species: Do They Play a Role in Adaptive Immunity?", *Frontiers in Immunology*, vol. 12, pp. 1–13, 2021.

[69] D. P. Jones, "Radical-free biology of oxidative stress", *American Journal of Physiology-Cell Physiology*, vol. 295, no. 4, pp. 849–868, 2008.

[70] L. Kuna, I. Bozic, T. Kizivat, "Models of Drug Induced Liver Injury (DILI) – Current Issues and Future Perspectives", *Current Drug Metabolism*, vol. 19, no. 10, pp. 830–838, 2018.

[71] A. Iorga, L. Dara, and N. Kaplowitz, "Drug-Induced Liver Injury: Cascade of Events Leading to Cell Death, Apoptosis or Necrosis", *International Journal of Molecular Sciences*, vol. 18, no. 5, pp. 1–25, 2017.

[72] A. B. Vliegenthart, C. S. Tucker, J. Del Pozo, and J. W. Dear, "Zebrafish as model organisms for studying drug-induced liver injury", *British Journal of Clinical Pharmacology*, vol. 78, no. 6, pp. 1217–1227, 2014.

[73] J. G. Stine and N. Chalasani, "Chronic liver injury induced by drugs: a systematic review", *Liver International*, vol. 35, no. 11, pp. 2343–2353, 2015.

[74] D. E. Kleiner, "Drug-induced liver injury: the hepatic pathologist's approach", *Gastroenterology Clinics*, vol. 46, no. 2, pp. 273–296, 2017.

[75] J. Poisson, S. Lemoinne, and C. Boulanger, "Liver sinusoidal endothelial cells: Physiology and role in liver diseases", *Journal of Hepatology*, vol. 66, no. 1, pp. 212–227, 2017.

[76] E. M. Brunt, A. Gouw, and S. Hubscher, "Pathology of the liver sinusoids", *Histopathology*, vol. 64, no. 7, pp. 907–920, 2014.

[77] A. Soeksmanto, P. Simanjuntak, and M. A. Subroto, "Uji Toksisitas Akut Ekstrak Air Tanaman Sarang Semut (Myrmecodia pendans) Terhadap Histologi Organ Hati Mencit", *Jurnal Natur Indonesia*, vol. 12, no. 2, 2012.

[78] N. Linasari, "Uji Toksisitas Sub Kronis Suspensi Keladi Tikus (Typhonium flagelliforme) Terhadap Gambaran Histopatologi Hati Tikus Putih (Rattus norvegicus) Jantan", Thesis, Universitas Airlangga, Surabaya, 2010.

[79] D. Isturiningrum, "Pengaruh Lama Pemberian Suspensi Herbal Keladi Tikus (Typhonium flagelliforme) Terhadap Kadar SGOT dan SGPT Pada Tikus Putih (Rattus norvegicus)", Thesis, Universitas Airlangga, Surabaya, 2010.