Effect of Soybean (*Glycine max*) Extract on Lymphocyte Count of Sheep Red Blood Cells (SRBC)- induced Mice (*Mus musculus* L.)

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Abstract— Autoimmune disease is one of the diseases with indefinitive cure and its therapy using immunosuppressants is still the mainstay until present time. Soybean (*Glycine max*) is one of many medicinal plants whose use is only for functional food. The stigmasterol compound is one of the compounds in soybeans that has the potential to be an immunosuppressant to inhibit the proliferation and differentiation of lymphocytes. The purpose of this study was to determine the stigmasterol content of soybean extract and its ability to modulate lymphocyte count in male BALB/c mice (*Mus musculus* L) as test animals. Local soybean cultivar was extracted by maceration using distilled water as a solvent. Stigmasterol content of soybean extract was analyzed using the HPLC method. To determine its modulation effect on leucocytes, soybean extract was given orally to mice at doses of 10, 50 and 100 mg/BW/day for 14 days. Mice were first injected using 0.1 ml of Sheep Red Blood Cells (SRBC) on day 0 and day 10 (booster) in all groups except negative control (K-) to induce immune responses. Pure stigmasterol was used as positive control. Lymphocytes were counted and observed by blood smear and differential count methods. The decrease in the value of lymphocytes indicates the effect and potential of stigmasterol in soybean extract to be an immunosuppressant. Based on this research, it is known that 0.023 mg/g stigmasterol was contained in soybean extract. Blood smear result showed that STG10 group had the lowest lymphocyte count (20.00/cell ± 8.00) and the K- group (48.00/cell ± 5.66) the highest. Based on the hemocytometer analyzer method, the highest lymphocyte count on day 24 was in the STG10 group (93.50/mm^3 ± 2.12) and the lowest was in the EK100 group (78.00/mm^3 ± 9.90). From these results, it can be said that the STG10 group is the most effective in suppressing the number of lymphocyte production in mice. Thus, stigmasterol in soybean extract has immunosuppressant potency to suppress lymphocytes count.

Index Terms— Soybean, Stigmasterol, Sheep Red Blood Cells, Mice, Lymphocytes

1 Introduction

The lifestyle of modern society moves quickly and instantaneously which inevitably makes the immune system of human body respond to this lifestyle. Nutrient-poor food, poor air quality, lack of exercise, and increased stress levels make the body vulnerable to pathogens such as viruses, bacteria, microbial parasites, and fungi which leads to disease, degenerative and premature aging.[1]. Approximately 3-5% of the world's population suffer from autoimmune diseases [2-4]. Autoimmune disease is a condition where the immune system is unable to maintain its stability, so the immune system perceives healthy body parts as foreign objects that must be destroyed. This abnormality is certainly very detrimental to the patient because healthy organs will be damaged [5-8].

The main cause of autoimmune disease itself is still specifically unknown that leads to unspecific medical treatment. However, there are several therapies employed to reduce the risk for patients, one of which is by inhibiting the production of lymphocytes. The inhibition of lymphocytes will be linear to the ability of Naïve T cells to proliferate and differentiate into specific CD8+ and CD4+ T cells that play role in eliminating antigens that infect human body (9,10). Research by Roffico and Djati (2014) on the effectiveness of ethanol extract of leaves of *Polyscias obtusa* and *Elephantopus scaber* on the modulation of CD4+ and CD8+ T cells in BALB/c pregnant mice stated that, based on the number of CD4+ and CD8+ T cells treated mice, the dose could not be determined. The optimum dose for increasing mice immunity was due to the potential of panaxadiol compounds in *Polyscias obtuse* and stigmasterol compounds in *Elephantopus scaber* as immunosuppressants (11). Asfi and Djati (2014) also revealed similar results in a study on the development of CD4 and CD62L T cells in the spleen of mice infected with *Salmonella typhimurium* after administration of ethanol extract of *Polyscias obtuse* and *Elephantopus scaber* leaves stated that the formula was 50%:50% of the initial dose. Fifty mg/KgBW provided an immunosuppressant effect and was able to increase the proliferation of nave CD4+ T cells (12). However, those researchers only assumed that stigmasterol compound contained in *Elephantopus scaber* when combined with the panaxadiol compound contained in *Polyscias obtuse* at a certain dose would have an immunosuppressant effect, so further research on the role of stigmasterol in immune suppression needs to be done.

Soybean (*Glycine max* L) is a plant that contains high content of stigmasterol. In this study, soybean was used because of it easiness to obtain and its highly consumption by Indonesian people. Indonesia itself places soybeans as the second largest food commodity after corn (*Zea mays* L) and has been widely used for household, industrial, and seed food consumption. In 2010, Indonesian soybean consumption reached 2.2 million tons per year [13,14]. Considering the large number of people contracted autoimmune diseases, it is very necessary to do research

on natural compounds that have the potential to become immunosuppressants. Therefore, this study was conducted to determine local soybean content of stigmasterol and its capability to modulate lymphocyte count in Sheep Red Blood Cells (SRBC)-induced mice (*Mus musculus* L).

2 METHODS

2.1 Preparation of Soybean Extract and Detection of Stigmasterol

Soybean (*Glycine max*) of Anjasmoro cultivar were purchased from local market in Yogyakarta, Indonesia. Dried simplicia was prepared by steps as follows: wet sorting, washing, oven drying, grinding, and sieving. A total of 105 g of simplicial was taken with an analytical balance and dissolved in 525 ml of solvent (distilled water) in a glass jar, then incubated for 24 h. After 24 h, filtered using a filter cloth to separate the filtrate and the dregs. Subsequently, powdered simplicia was macerated by adding 525 ml of solvent and incubated again for 24 h. The process is repeated for 3 times. Filtrate obtained from the first to the third maceration was concentrated using a vacuum rotary evaporator (IKA RV-8) at a temperature of 70^{o}C. The thick extract was then put in an oven (MEMMERT Model 30-1060) at 40^{o}C to form a paste.

Detection of stigmasterol was carried out by HPLC analysis using liquid chromatography at the Integrated Chemistry Laboratory, Universitas Kristen Satya Wacana, Salatiga. The stationary phase used was Eurospher 100-5 RP C-18 150x4.6 mm column (KNAUER), and distilled water stationary phase (99:1, v/v). The flow rate is 1 ml/min at 30oC. Detection wavelength was set at 254 nm UV/VIS. Positive control used in this study was pure Stigmasterol from Wellnature Biotech Co., Ltd Shaanxi China.

2.2 Preparation of 1% Sheep Red Blood Cell Suspension

The preparation of 1% sheep red blood cell (SRBC) as antigen suspension, purchased from PT. Gajah Mungkur Indotama, Indonesia, was carried out at the Biochemistry Laboratory, Universitas Kristen Duta Wacana, Yogyakarta. The sheep blood obtained was put into a falcon tube and centrifuged at 3000 rpm for 10 minutes. Formed supernatant was discarded and PBS solution pH 7.2 was addes onto the falcon tube, then centrifuged at 300 rpm for 10 minutes. This washing step was repeated three times. At the end of washing step, 100% SRBC was obtained. To obtain 50% of SRBC suspension, 0.5 ml of 100% SRBC stock suspension was pippeted to be added with 50 ml of PBS. To prepare 1% SRBC suspension as working solution, 1 ml of the 50% SDMD suspension was taken, and 50 ml of PBS was added to falcon tube [15,16].

2.3 Acclimatization and Treatment of Test Animals

Test animals employed were male BALB/c mice (*Mus musculus* L) aged 6 weeks with weight range of 25-35 g. Mice were obtained from the Tikus Lovers Jogja Breeder, Yogyakarta. Prior to use, acclimatization was carried out for 7 days in cages. This research has received an ethical clearance from Health Research Ethics Commission, Faculty of Medicine, Universitas Kristen Duta Wacana in the Statement of Ethical Eligibility Number: 1313/C.16/FK/2021 issued on June 29, 2021. Twenty-four mice were divided into 8 groups with following group descriptions shown in Table 1. Mice were kept in 30 x 35 x 11 cm-dimension cages, where one cage dwelled by 3 mice.

Mice from treatment groups of K+, EK10, EK50, EK100, STG10, STG50, STG100 were induced by 0.1 ml of 1% SDMD intraperitoneally, while the mice group K- was induced with 0.1 ml sterile distilled water in the same way. The day of injection was counted as day 0. On the 9th day, the number of mice lymphocytes was counted using blood smear method. On the 10th day, as a booster, mice were re-injected with 1% SDMD and sterile distilled water using the same procedure. On the 10th day, mice were given soybean extract treatment according to their respective groups and doses. Doses of 10, 50, and 100 mg/BW per day were given in liquid form (dissolved in distilled water with the composition in Table 1) as much as 0.5 ml orally. The oral treatment of soybean extract was given every day until the day of 24th. On the 18th and 24th days (before euthanasia), number of mice lymphocytes was counted using blood smear method, followed formula described by Arif (2009) [17].

$$\text{Numbers of Cell} = \frac{Expected\ \ Number\ \ of\ Cell\ Types}{100\ leukocytes} \text{ x } 100$$

On the 24th day, mice were sacrificed by inhalation of overdosed chloroform and blood was drawn from the heart vena cava and put into the EDTA K3 blood container for differential count analysis using a hemocytometer analyzer as comparison to blood smear count. Hematology analysis was carried out at the SADEWA Clinical Laboratory, Yogyakarta.

TABLE 1
TREATMENT GROUP AND DOSE

Groups	Description
Control – (K-)	Distilled water induction, distilled water oral treatment.
Control + (K+)	SRBC Induction 1%, distilled water oral treatment.
EK10	SRBC Induction 1%, oral treatment of soybean extract 10 mg/BW/day
EK50	SRBC Induction 1%, oral treatment of soybean extract 50 mg/BW/day

EK100	SRBC Induction 1%, oral treatment of soybean extract 100 mg/BW/day
STG10	SRBC Induction 1%, oral treatment of stigmasterol 10 mg/BW/day
STG50	SRBC Induction 1%, oral treatment of stigmasterol 50 mg/BW/day
STG100	SRBC Induction 1%, oral treatment of stigmasterol 100 mg/BW/day

**EK (Ekstrak Kedelai) which means Soybean Extract. STG (Stigmasterol*

3 Result and Discussion

3.1 Soybean Extract and Detection of Its Stigmasterol Content

From 2.5 kg of simplicia obtained 450g of thick brown soybean extract in distilled water solvent. There was a depreciation of 82%. This shrinkage is not much different from the research of Djamil and Anelia (2009) where from 100g of simplicia obtained 22g of extract, which means there is a shrinkage of 78% [18]. The obtained paste was then analyzed to determine its stigmasterol content using HPLC with pure stigmasterol as a comparison. It was found that the stigmasterol content in our soybeans extract with water as solvent was 0.023 mg/g. (Table 2). This stigmasterol content is relatively small if compared to research conducted by Slavin and Yu (2012) who found 0.23 mg/g of stigmasterol. This difference was probably caused by different solvent used to extract stigmasterol. In their study, Slavin and Yu (2012) employed a combination of acetonitrile, methanol, and water. The use of polar compounds as solvents in stigmasterol extraction is indeed quite good considering stigmasterol compounds are also classified as polar [19-22].

However, distilled water was still used in this study to minimize the risk of the effects of using organic solvents such as methanol and acetonitrile, petroleum ether, and dichloromethane, which would result in continuous stimulation of the mice's immune system [23-26]. On the other hand, the use of non-organic solvents is also feared to have a stimulating effect on the immune system of mice due to inflammation in the organs of mice.

TABLE 2
CALCULATION OF THE AMOUNT OF STIGMASTEROL BASED ON THE RATIO OF THE SAMPLE AREA TO THE STANDARD AREA OF STIGMASTEROL

Concentration (mg/ml)	Area	[µg/ml] Test	[mg/g] Sample
2,5	550	0,057	0,023

the standard area of stigmasterol 0.1 g/ml = 7818

3.2 Effect of 1% SRBC Induction on Mice Immune Response

In this study, exogenous antigen was administrated in the form of 1% SRBC to stimulate the immune response of mice. The use of SRBC was based of its easiness to obtain, its uniform suspension and its high antigenic properties [27,28]. SRBC was also chosen because of its easy handling when compared to bacteria as antigens. Risk factors are also considered, considering the risk of using bacteria is higher than that of SRBC, because bacteria have the potential to exert long-term inflammatory effects on the body.

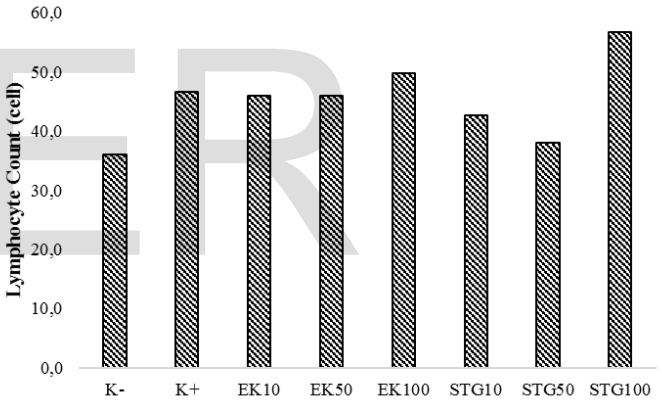

Figure 1. *Lymphocytes count in the blood on day 9 by blood smear method.*

Immune response of mice induced by SRBC was successful. As shown in Figure 1 which informed lymphocytes count on 9th day, group K- had the lowest lymphocyte count of 36 cells/cell ± 5.29, while another group of mice induced by SRBC had a lymphocyte count above 40 and the highest was STG100 group (56.7 cells/cell ± 25.48). This means that SRBC (foreign substances) can effectively stimulate the immune system. Suhirman and Winarti (2010) similarly mentioned that foreign substances that are not derived from the body of mice will cause an increasingly effective immune response, the more foreign antigens used, the more effective the immune response will be [5]. The same result was also stated by Lis et al (2011) which stated that the control treatment mice (not induced by SRBC) had a value of 34.6 ± 4.6 [28].

3.3 Effects of SE and STG on Lymphocyte Count Using Blood Smear

The first method to count lymphocyte was blood smear (Figure 2). The highest number of lymphocytes using the

blood smear method on day 24 was in the K- group (48.00/cell ± 5.66) and the lowest was in the STG10 group (20.00/cell ± 8.00) (Figure 3). In a bigger picture, all treatment doses involved SE and STG tend to decrease lymphocytes count. In contrast, the K- group experienced an increase in the number of lymphocytes every week, while the lymphocyte count of the K+ group tended to be stable as seen from the small difference between the measurement results.

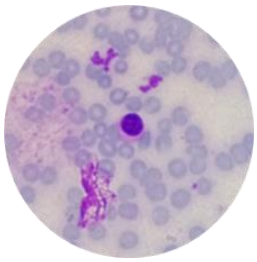

Figure 2. *Lymphocytes observed by blood smear method.*

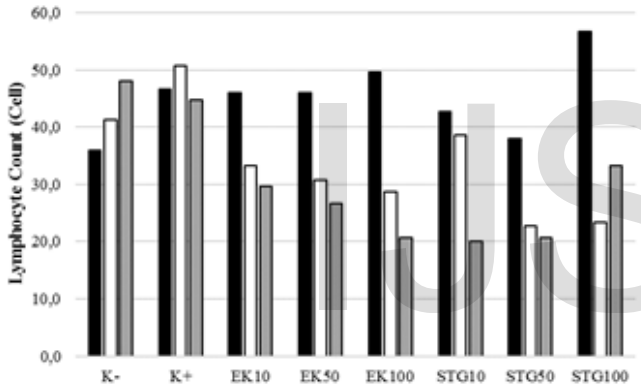

Figure 3. *Lymphocytes count using blood smear method. Black chart of lymphocyte count on day 9, white chart for number of lymphocytes on day 18, gray chart for number of lymphocytes on day 24.*

Reviewing the effect of SE and STG treatment on the number of lymphocytes using the blood smear method, there were 3 treatment groups that had a significant effect on suppressing lymphocytes. The three groups, respectively, the best was STG 10 (20.00/cell ± 8.00), STG 50 (20.67/cell ± 8.08), and SE 100 (20.67/cell ± 10, 07). The same thing was expressed by Le *et al* (2017) which stated that sterols and stigmasterol were able to suppress the relative proliferation rate of Concanavalin A (Con-A)-induced mice. This is because the affinity of stigmasterol binds to T cell receptors (T Cell Receptor, TCR) on T lymphocytes but not on Toll-like receptors (TLRs)-4 and myD88/MAL/TRIF/TRAM which is the mediating pathway on B lymphocytes [29]

This binding results in inhibition of T lymphocytes both competitively and non-competitively with Con-A against TCR. In addition, stigmasterol can also inhibit the final TCR-mediated pathway, thereby reducing T cell activity and cytokine release [30]. Thus, the higher the dose of treatment extract, the greater its potential in suppressing the rate of lymphocyte production. This is different from the dose of pure stigmasterol treatment, where the STG100 treatment group did not have a lymphocyte suppressive effect on day 24. This was possible because the dose given was too high so that it could not be completely absorbed by the mice. Complete absorption of compounds by mice will provide optimal effects. Complete absorption of 10 mg of stigmasterol compound was indicated by the largest liver weight.

3.4 Effects of SE and STG on Lymphocyte Count using Differential Count

As a comparison to blood smear method, a differential count using hemocytometer analyzer was employed to count total lymphocytes. Based on differential count, the highest lymphocyte count on day 24 was in the STG10 group (93.50/mm^3 ± 2.12) and the lowest was in the SE100 group (78.00/mm^3 ± 9.90) (Figure 4). Statistical analysis of differential count result also showed no significant difference in the number of lymphocytes between each group. The difference in results could be influenced by more influencing factors in the hematological analysis. These factors are more related to sample handling such as temperature, duration of sample storage, and the type of anticoagulant used.

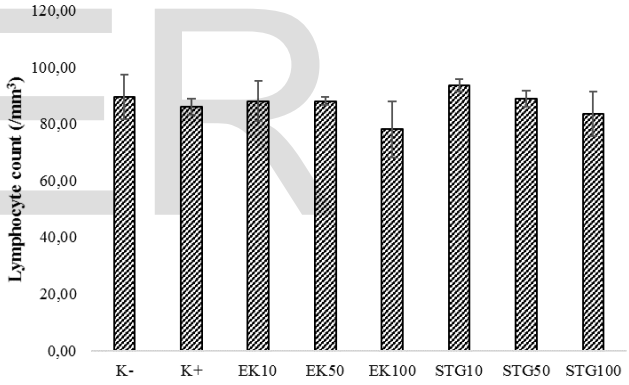

Figure 4. *Lymphocytes counts using differential count method on day 24*

Fitria *et al* research (2017) revealed that the longer the sample stored, even at 4^{o}C, the lower the total number of leukocytes, neutrophils, lymphocytes, and monocytes. The study clearly stated that the number of lymphocytes stored at 4^{o}C for 48 hours would decrease by 1.12 x 10^3/mm^3 [31]. In this study, analysis of blood samples using a differential count was carried out 21 days after taking blood from the heart. This long-term storage of blood sample was due to mobility restriction on Covid19 Pandemic in Yogyakarta. Fahrimal *et al* (2014) stated that under normal conditions, the percentage of lymphocytes in mice ranges from 55-95% of the total leukocytes [32]. Lymphocytes count of the K+ and K- groups in this study did not meet the standard of 55-95% of total leukocytes because the percentage were only 0.8% (K+) and 1.3% (K-) (Table 3).

Because of the obstacle for counting lymphocyte using dif-

ferential count, data on the lymphocyte count on this study was mainly based on blood smear method. From the blood smear method, all treatment groups tended to suppress lymphocyte production in mice, which was seen from the decreasing trend from day 9 to day 24. There is one exception in this case where the STG100 group increased on the 24th day for the reasons mentioned in the discussion above.

TABLE 3
DISTRIBUTION OF THE PERCENTAGE OF THE NUMBER OF LYMPHOCYTES TO THE TOTAL NUMBER OF LEUKOCYTES

Groups	Lymphocyte Percentage (%)
K-	1,32
K+	0,83
EK10	1,10
EK50	1,09
EK100	1,05
STG10	0,26
STG50	0,82
STG100	0,99

4 CONCLUSIONS

Stigmasterol content of soybean extract with distilled water as solvent was 0.023 mg/g. Soybean extract dose of 100 mg/BW per day was the best dose in suppressing the lymphocytes count.

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