Total Phenolic Content and Proliferation Activity of Spirulina Extract in Lymphocyte Cell

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Abstract. Decreased immunity can make the body susceptible to diseases. Many side effects due to the use of drugs such as chemotherapy drugs cause many people to use natural ingredients to be used as supplements. Lymphocyte cell proliferation is cell division or multiplication to enhance the immune system. This study aims to determine the total phenolic content of the 70% ethanolic extract of *Spirulina platensis* and to analyze the potential of this extract to increase the proliferative activity of lymphocyte cells. The measurement of total phenolic levels used the Folin-Ciocalteu method. The measurement of the viability of lymphocyte cell proliferation activity used the MTT method. The 70% ethanolic extract of *S. platensis* in this study had a total phenolic content of 2.4957±0.0597 GAE mg/g. The 70% ethanolic extract of *S. platensis* increased the lymphocyte cell proliferation activity. The best viability results were found at a concentration of 20 ppm of 124.89±1.84%.

1 Introduction

The immune system is a mechanism in living bodies to protect the body against pathogenic infections, especially during a pandemic, by identifying and killing them [1]. Every day the body can be contaminated with various things that trigger the immune system to work. Decreased immunity can make the body susceptible to disease. Things that can cause a decrease in immunity are genetics, physiology, stress, age, hormones, exercise, lack of rest, unfulfilled nutrition, and exposure to hazardous substances such as radioactive, cigarettes, pesticides, and other chemicals such as alcohol [2]. Common diseases due to decreased immunity not only cause fever, runny nose, sneezing, coughing, and sore throat, but can also cause malignant diseases such as cancer [3].

Immunity can be increased by consuming a nutritious and balanced diet, exercising, reducing stress, improving the digestive system or hormones, and taking health supplements [2]. The number of side effects due to the use of hard drugs such as chemotherapy drugs causes many people to use natural ingredients to be used as supplements. Natural ingredients are considered to have fewer side effects than chemical drugs [4]. Health supplements are products used to supplement nutritional needs, maintain, improve and/or improve health functions, have nutritional value and/or physiological effects, contain one or more ingredients

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in the form of vitamins, minerals and amino acids [5]. One of the natural ingredients that has been used as a health supplement that has various benefits is *Spirulina platensis.*

Spirulina platensis is a microalgae belonging to the *Cyanobacteria* group which contains phycocyanin, so it tends to be blue-green in color. Spirulina is known to contain high protein, carotenoids, as well as a source of micronutrients that are beneficial for health [6]. Spirulina is thought to be used as an immunomodulator to increase body immunity because it has immunostimulating activity. Immunostimulants are special compounds that can trigger the body's defense system non-specifically and specifically [7]. Compounds from natural ingredients such as flavonoids are thought to have immunostimulating properties because they can increase the synthesis of IL-2 resulting in the activation and proliferation of T lymphocyte cells [8]. Flavonoids are phenolic group compounds which are found in many natural products [9]. [10] stated that spirulina methanol extract contains flavonoids. Research [11] also stated that the ethanol extract of spirulina contains flavonoid compounds. So that flavonoids are thought to have a role in spirulina activity as an immunomodulator.

Lymphocyte proliferation is the first step in the immune response to create effector lymphocytes or memory lymphocytes [12]. Lymphocyte cells are found in secondary lymphoid organs which belong to the specific immune system. The specific immune system is an adaptive immune system that is specific or able to differentiate and recognize pathogens. Specificity causes the reaction of specific lymphocyte cells against certain pathogens. The specific immune system is carried out by B, T, and Antigen Presenting Cell or APC lymphocyte cells [13]. To determine the potential activity of spirulina in increasing lymphocyte cell proliferation, you can use the colorimetric microtetrazolium (MTT) assay method by reading the absorbance of the formazan produced using spectroscopy, with the results used to measure the magnitude of lymphocyte cell viability [14].

Previous research conducted by [10] showed that the total phenolic content of the methanol extract of *Spirulina platensis* was 21.93±1.79 GAE mg/g. According to [14], *Spirulina platensis* extracted using methanol has potential as an antioxidant because it has an MDA (malonedialdehyde) inhibitory power of 56.53%. Research related to the total phenolic content of 70% *Spirulina platensis* ethanol extract and its effect on lymphocyte cell proliferation activity has never been carried out, so this study aims to determine the total phenolic content of 70% *Spirulina platensis* ethanol extract and analyze the potential of this extract to increase lymphocyte cell proliferation activity to find alternatives immunomodulator.

2 Materials and methods

2.1 Materials

The materials used in the preparation of the extract were *Spirulina platensis* dry powder obtained from Jepara, 70% ethanol, distilled water, Follin-Ciocalteu reagent (Merck), Na_2CO_3 7.5% CAS 497-19-8 (Merck), gallic acid CAS 149-91- 7 (Merck), Phosphate buffered saline (PBS) (Gibco, USA), ficoll, tube with anticoagulant (Heparin), Roswell Park Memorial Institute medium (RPMI), 3% acetic acid, isopropanol, and 3-(4,5)-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide reagent (MTT) (Sigma, USA).

2.2 Extraction of Spirulina [15]

Extraction of *Spirulina simplicia* was carried out by maceration method using 70% ethanol solvent. Extraction was carried out by mixing dry simplicia with a solvent with a ratio of 1:10 (w/v). The simplicia used was 5 grams dissolved in 50 mL 70% ethanol. The simplicia and

solvent mixture was macerated using a shaking incubator at 125 rpm at room temperature for 24 hours. Remaceration was carried out 5 times (until the color of the mixture turned faded). The maceration results were filtered using a vacuum to produce a filtrate. The filtrate was concentrated to form a paste using a rotary evaporator with a temperature of 50°C. The yield obtained is calculated using the following equation:

$$Yield = \frac{Extract\ weights\ (g)}{Simplicia\ weight\ (g)} \times 100\% \quad (1)$$

2.3 Measurement of total phenolic content [16]

Preparation of Standard Solutions. The standard used in this study was gallic acid solution. Gallic acid solutions were made with various concentrations, namely 5, 10, 15, 20, 25, 30, 35 and 40 µg/mL. A total of 300 µL of gallic acid solution was taken from each concentration and put in different tubes. A total of 1.5 mL of 10% Folin Ciocalteau reagent was added to each tube, then stirred. The mixture was incubated in a dark room for 8 minutes, then 1.2 mL of 7.5% Na_2CO_3 solution was added to each tube. The mixture was incubated again for 30 minutes in a dark room. The absorbance of each solution was measured at a wavelength of 765 nm using a spectrophotometer. The standard value of gallic acid is expressed as gallic acid equivalent (GAE mg/g or milligrams of extract concentration per gram of sample).

Measurement of Total Phenolic Levels. A total of 300 µL of Spirulina extract solution was taken and 1.5 mL of Folin-Ciocalteu reagent was added. Mixture allowed to stand for 3 minutes, then added 7.5% Na_2CO_3 solution as much as 1.2 mL. The mixed solution was incubated for 30 minutes inside dark room and measured the absorbance value at length 765 nm using a UV-Vis spectrophotometer. The total phenolic content of Spirulina extract is expressed as GAE mg/g.

$$Total\ fenolic\ GAE = measurable\ phenol\ concentration\left(\frac{mg}{L}\right) \times \frac{1}{extract\ concentration\ (mg/L)} \quad (2)$$

2.4 Lymphatic cell proliferative activity [17]

Lymphocyte Cell Preparation. Blood samples were prepared for separation of single nucleated blood cells. Prepare a centrifuge tube with lithium anticoagulant heparins. Samples were put into the same tube to be centrifuged at 1500 rpm for 10 minutes. The results of centrifugation will separate into several layers, namely plasma, buffy coat and red blood cells. The plasma layer was carefully removed using a pipette. Buffy coat was taken as much as 1 mL, then diluted with 4 mL of PBS 1x. A 15 mL centrifugation tube was prepared and 5 mL of ficoll solution was added, then carefully diluted buffy coat was added to form a layer. The mixture was centrifuged at 2700 rpm for 30 minutes.

The lymphocyte cell layer was taken using a transfer pipette and diluted with PBS 1x as much as 5 mL. Lymphocytes that had been diluted were centrifuged at 2000 rpm for 15 minutes. The pellets from the centrifugation results were taken and 1 mL of RPMI media was added. The resulting single-nucleated cells were counted by adding 10 µL of cell suspension and 90 µL of 3% acetic acid. Cells were grown in 96 wells of tissue culture plate with a concentration of 100,000 µg/mL for each well, then incubated for 18-20 hours at 37 ºC and 5% CO_2. Lymphocyte cells that have been cultured are added to the sample at the desired concentration. The mixture was incubated for 48 hours at 37ºC and 5% CO_2.

MTT Test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Lymphocyte Cells. Lymphocyte cells that have been cultured and added to the sample prepared. A total of 50 µL of MTT reagent was added to each wells, then incubated at 37ºC and 5% CO_2 for 4 hours. The mixture will produce formazan crystals. Crystal formazan was

dissolved by adding 1N HCl in isopropanol 100 µL per well. The mixture is read absorbance using a microplate reader at length 595 nm wave. The results obtained are in the form of optical density values (OD). Percent viability of lymphocyte cells is calculated by the following formula following.

$$\%Viability=\left(1-\frac{(OD\ control\ cells-OD\ treatment\ cells)}{OD\ control\ cells}\right)\times 100\% \quad (3)$$

2.5 Data analysis

The results of the lymphocyte cell MTT test were analyzed using the test analysis of variance (ANOVA) test with a 95% confidence level and a level of α=0.05. Test ANOVA was performed using the SPSS program to see real differences sample.

3 Results and discussion

3.1 Yield of spirulina extract

Spirulina platensis extract was obtained by extracting the sample using the maceration extraction method. Maceration was carried out using 70% ethanol for 24 hours and repeated maceration (remaceration) was carried out 5 times. Remaceration is carried out until the color of the concentrated solution turns pale or clear. The yield was obtained based on the ratio of the weight of the extract, which was 1.52 grams to the weight of the simplicia, which was 5.01 grams. The yield obtained from the spirulina extraction process with 70% ethanol solvent is 30.32%.

The resulting yield value indicates the amount of bioactive compounds contained in the sample. Bioactive compounds have many benefits such as being antibacterial, anti-inflammatory, anticancer and can be a source of antioxidants [18]. Spirulina extraction using 70% ethanol and remaceration 5 times resulted in a yield of 30.32%. The longer the maceration time can cause the cell wall to break down in the sample resulting in the diffusion of the solute into the solvent. Therefor samples with longer extraction times will have higher yield values. The length of extraction time is important to note so as not to exceed the optimum time. Extraction that passes the optimum time can cause the dissolved substances in the material to be damaged and the potential for evaporation of compounds in the solution [19].

According to [20] spirulina extraction uses 96% ethanol solvent and for 3x24 hours produces a yield of 2.36%. These results are smaller than extraction using 70% ethanol solvent. This is because the higher the solvent concentration, the lower the polarity, besides that the length of extraction time can also affect the yield value produced. According to [21] the amount of ethanol concentration is inversely proportional to the yield produced. The high concentration of ethanol will reduce its polarity as a solvent so that the ability of ethanol to extract polar compounds will also decrease.

3.2 Total phenolic content of spirulina extract

The total phenolic content in the 70% ethanol extract of spirulina was measured using the Folin-Ciocalteu method with gallic acid as standard. Standard measurements of gallic acid produce a line equation y=0.0657x+0.0247 with an R^2 value of 0.9988. The correlation value (R^2) between 0 – 1 proves that the regression equation is linear. The closer to 1 the R value is obtained, the better the resulting correlation. The total phenolic content of the samples was

measured at a concentration of 2000 ppm with 3 repetitions of the test. The average measurement results for the total phenolic content of spirulina samples with 70% ethanol solvent were 2.4957±0.0597 GAE mg/g.

Phenolic compounds can generally be found in plants either in the form of simple phenols or polyphenols. These compounds have an aromatic ring with one or more hydroxyl groups [22]. Analysis of total phenolic content in spirulina extract was carried out to determine the amount of phenolic compounds present in the sample. Tests were carried out using the Folin-ciocalteu method. The principle of this method is the formation of a blue colored complex due to the oxidation of the hydroxyl groups in the phenolic as measured using a UV spectrophotometer. The reagent will oxidize the phenolic, then reduced to a blue molybdenum-tungsten (Mo-W) complex. The intensity of the blue color indicates the amount of phenol content in the sample. The more concentrated the color of the sample, the greater the concentration of phenolic compounds contained in the sample [23]. Gallic acid is used as a standard solution, because gallic acid belongs to the class of phenolic compounds derived from hydroxybenzoic acid which are stable, simple and easy to find. Gallic acid is known to have antioxidant activity and has a higher reactivity to the Folin-ciocalteu reagent than other phenolic compounds [24].

Spirulina extract with 70% ethanol yielded a total phenolic content of 2.4957±0.0597 GAE mg/g. At the same concentration, namely 2000 ppm, the total phenolic content of the spirulina extract with 70% ethanol was smaller than that of the methanol extract of spirulina, which was 21.93±1.79 GAE mg/g [10]. The length of extraction time is known to affect the value of phenolic content in an extract. The longer the extraction time, the longer the contact between the solvent and the extract, so the process of dissolving phenolic compounds will continue until the solvent is saturated. Optimum extraction time is important to note in order to prevent exposure of the extract to oxygen. Extraction that exceeds the optimum time causes the possibility of oxidation of phenolic compounds, so that the extracted phenolic compounds will decrease [25].

The type of solvent is known to affect the value of the total phenolic content of the extract. Each solvent has a different level of polarity, a high total phenolic value indicates that the solvent has the same polarity level as the compounds in the extract [26]. Ethanol solvent produces less total phenolic compared to methanol solvent, this shows that methanol solvent is a better solvent in extracting phenolic compounds in Spirulina than ethanol solvent. Both methanol and ethanol are semi-polar solvents, but methanol is more polar so that phenolic compounds dissolve more easily in methanol solvents. The phenolic compounds present in the spirulina samples are thought to have broad polarity values so that the phenolic compounds can dissolve in ethanol and methanol solvents [27].

There are several factors that influence the extraction of phenolic compounds, namely the extraction method, solvent, extraction time, number of samples used and particle size of the simplicia [28]. Phenolic compounds are known to have antioxidant activity, this is due to their ability to reduce reactive oxygen. Phenolic compounds can absorb reactive oxygen because they have aromatic rings with several hydroxy groups that act as hydrogen donors. The total phenolic content value is directly proportional to the antioxidant activity. The higher the total phenolic content in the sample, the higher the free radical scavenging activity [29].

3.3 Lymphocyte cell proliferative activity

Lymphocyte cell proliferation activity was tested by the MTT method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. The results obtained are in the form of optical density (OD) values with the final result in the form of percent viability. Figure 1 shows the highest average percent viability value obtained from a concentration of 20 ppm of 124.89±1.84%. The average OD value produced is directly proportional to its

viability, the higher the OD value, the higher the viability value. The results of the normality test were obtained from 8 concentrations, all of which had a p value >0.05, so it was known that the viability data for lymphocyte cell proliferation activity of spirulina extract were normally distributed. One-way ANOVA test results sig. sample was 0.000 with p<0.05 meaning that concentration data had a significant effect on viability.

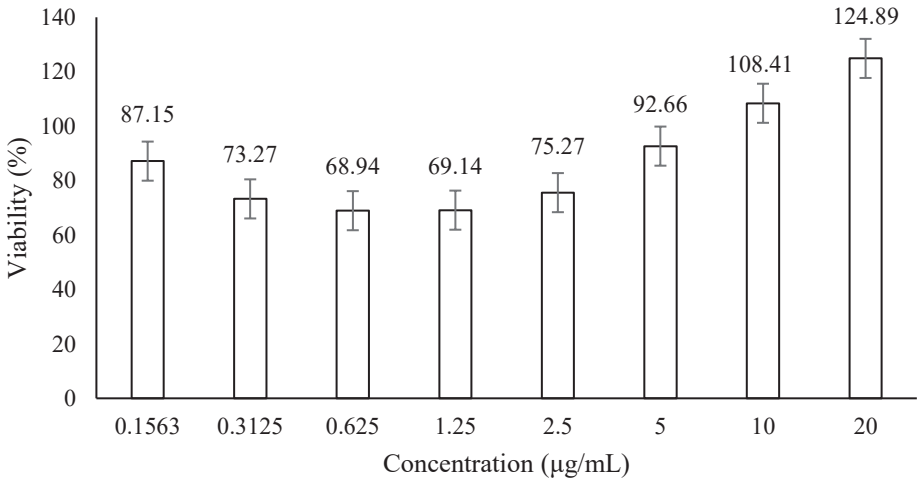

Fig. 1. Lymphocyte cell viability value of spirulina ethanol extract.

Immunomodulators are substances that can modulate or influence the immune system in the body so that it can work better [30]. Immunomodulators are divided into three types, namely immunostimulators, immunosuppressors and immunoregulators. Immunostimulators are substances that function to increase the function and activity of the immune system. Immunosuppressors are substances that can inhibit or suppress the activity of the immune system. Immunoregulators are substances that play a role in regulating the immune system [31]. Many compounds from natural products are known to be immunostimulators, for example flavonoids. Flavonoids are known to act as immunostimulators because they can increase the activity of the immune system, namely by increasing IL-12 activity and lymphocyte cell proliferation [32].

Lymphocytes are part of the white blood cells which are very numerous in the body, consisting of T cells and B cells. Lymphocytes play an important role in the immune system for defense against pathogens [33]. The number of lymphocytes in the body is around 20-40% of total leukocytes, the second most from neutrophils, around 50-70% [34]. Cell proliferation is a process of maturation and cell multiplication through cell division (mitosis) [35]. So that the proliferation of lymphocyte cells is a process of maturation and multiplication of lymphocyte cells to enhance the immune system. The process of cell proliferation can occur when there is exogenous stimulation from the active ingredients [36].

An immunomodulatory substance can be considered as an immunostimulator if it contains compounds capable of increasing the response of the immune system, in this study in the form of increased activity of lymphocyte cells due to the administration of *Spirulina platensis* ethanol extract. Lymphocyte cell proliferation activity due to mitogen administration can be seen from the results of OD uptake or absorbance. The OD (Optical density) value is the value of optical density based on turbidity which shows the growth of the cell population compared to blanks using a spectrophotometer [37]. The resulting OD value or absorbance value indicates high or low growth or cell population in a medium [38]. In this study, the high OD value indicated high cell proliferation activity as well. The OD

value is directly proportional to the viability value, the higher the OD value the higher the viability value. Viability is the percentage value of cell survival obtained from the OD value of the sample absorbance reading [16].

Testing the proliferative activity of lymphocyte cells was carried out using the MTT method. The principle of this method is the breakdown of the yellow methylthiazole tetrazolium salt into formazan crystals which are blue-purplish in color as a result of a cellular reduction reaction. The splitting of MTT salts into formazan crystals occurs due to the presence of the enzyme succinate dehydrogenase in the mitochondria of living cells. The breakdown reaction involves pyridine nucleotide cofactors NADH and NADPH which are only catalyzed by living cells, therefore the number of formazan crystals formed is directly proportional to the number of living cells. The more cells that are alive, the more formazan crystals are formed which can also be indicated by the darker purple color shown after the addition of HCL-isopropanol [39]. The addition of HCl isopropanol to cell culture during testing serves to dissolve formazan blue crystals and decompose lymphocyte cells [40].

The results of the MTT test on lymphocyte cells showed that spirulina extract could increase lymphocyte cell proliferation. All of the eight extract concentrations tested showed that spirulina extract could increase lymphocyte cell proliferation, which could be seen from the absence of negative results in percent viability (Figure 1). The highest lymphocyte cell proliferation was produced at a concentration of 20 ppm of 124.89 ± 1.84%. These results are consistent with the research of [41] who stated that spirulina could increase lymphocyte cell proliferation, spirulina could increase the production of CD4+ T lymphocytes by 35.98% and CD8+ T lymphocytes by 20.50%. T lymphocytes differentiate into CD4+ and CD8+. CD4+ T cells are commonly known as helper T cells because they can signal B cells to produce antibodies and help phagocytic cells destroy pathogens [46]. CD8+ T cells or cytotoxic cells are cells whose function is to kill cells that have been infected with antigens [42].

Spirulina platensis can increase lymphocyte cell proliferation allegedly because it contains polysaccharides and phycocyanins which act as immunostimulants [43] in [44]. [45] in [46], stated that polysaccharides can stimulate macrophages and T and B cell proliferation, then activate these cells to secrete TNF-α, IL -1 β, IFN γ, IL-2, and IL-6. The polysaccharides in spirulina are thought to possibly bind to the mannose receptors (MR) of dendritic cells and macrophages which will then initiate the activation of immunity. According to [47] in [48] phycocyanin can affect immunity because it can secrete TNF- α, IL-1 β, and IL-6.

Spirulina is also known to contain flavonoid compounds, these compounds are polyphenolic compounds in the phenolic group which are thought to enhance the immune system by increasing IL-2 production, increasing lymphocyte cell proliferation and can affect CD4+ cells [10, 49].

4 Conclusion

The 70% ethanol extract of *Spirulina platensis* in this study had a total phenolic content value of 2.4957±0.0597 GAE mg/g. *Spirulina plantensis* 70% ethanol extract can increase lymphocyte cell proliferation activity. Lymphocyte cell proliferation activity was seen from cell viability. The best viability value was found in the 70% ethanol extract of *Spirulina platensis* with a concentration of 20 ppm, which was 124.89±1.84%.

Suggestions that can be given based on the results of this research evaluation are necessary extraction of samples with different solvent concentrations is carried out in order to be able to a comparison of results is carried out. In addition, the optimum extraction time should be more pay attention so that the extracted secondary metabolites are maximized.

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