

In Vitro Immunomodulatory Activity of Fig Fruit Ethanol Extract (*Ficus carica* Liin) Against Phagocytosis Macrophages and Lymphocyte Proliferation

Iis Nur Azizah, Aji Winanta*

School of Pharmacy, Faculty of Medicine and Health Sciences
Universitas Muhammadiyah Yogyakarta

ABSTRACT

Fig (*Ficus carica* L.) is a natural product that potentially can improve the immune system because it has flavonoids that have the potential as immunostimulants. The research aims to determine the possibility of fig fruit ethanol extract as an immunomodulator. Immunomodulatory activity is determined by knowing the activity of macrophage phagocytosis and lymphocyte proliferation in vitro and the levels of flavonoids in the extract. The research began with extraction, and then the sample was tested with TLC and colorimetry methods. Furthermore, the immunomodulatory activity test sample in vitro was measured through the activity of macrophage phagocytosis and lymphocyte proliferation. In the phagocytosis activity test, macrophage cells were given samples in various concentrations and latex beads. The number of activated macrophages and the number of latex phagocytosed by the macrophage is then calculated. For tests of lymphocyte proliferation activity, lymphocyte cells were sampled with different concentrations and induced hepatitis B vaccine. Then the cell absorbance was read with an Elisa reader at 550nm wavelength. The study results found that the samples contained flavonoid compounds, and the total flavonoid levels obtained were 0.74 ± 0.01 mgEQ/g samples. The immunomodulatory activity showed that the sample increased phagocytosis activity of macrophages compared to cell control. The lymphocyte proliferation test produced stimulation index < 2 values, showing no effect on the proliferation of lymphocytes. This study indicated that fig fruit ethanol extract could increase the phagocytosis activity of macrophage cells but did not affect the proliferation of lymphocyte cells in vitro.

Keywords: Fig fruit; total flavonoids; phagocytosis macrophages; lymphocyte proliferation; in vitro

INTRODUCTION

Indonesia is an archipelagic country that has a lot of flora and fauna. In Indonesia, there are about 30,000 species of plants, of which 7,000 species have medicinal properties (Jumiarni & Komalasari, 2017). The use of natural medicines that have the potential as immunomodulators is one of the new concepts in traditional medicine during this pandemic. An immunomodulator is a compound in the body that can affect the immune system. In general, immunomodulators are divided into two categories, namely immunosuppressants and immunostimulants. Immunosuppressants are compounds that can suppress excessive immune responses and be used for autoimmune diseases. In addition, immunostimulants are compounds that work to increase the lack of immune response (Krensky *et al.*, 2006).

One plant with potential as an immunomodulatory agent is fig (*Ficus carica* L.). Fig plant (*Ficus carica* L.) is a plant that has many benefits. One of them is the part of the fruit used to treat gastrointestinal and respiratory

tract diseases, anti-inflammatory, and boost the immune system. The core components of the fig plant contain flavonoids, terpenoids, polyphenols, alkaloids, and tannins. Flavonoids can improve the body's defense system because they can increase the production of IL-2, which is involved in the activation and proliferation of lymphocyte cells (Carmelit., 2016), and can affect CD4+ cells, then cause Th1 cells to be activated. Activated Th1 cells will affect SMAF (Specific Macrophage Activating Factor); multiple molecules, including IFN, can activate macrophages (Baratawidjaja and Rengganis, 2012). Based on this, the researchers wanted to know the immunomodulatory activity of fig fruits by looking at the phagocytic activity of macrophages and lymphocyte proliferation.

METHODOLOGY

Materials

The research material used was fig fruit (*Ficus carica* L.) taken from the Sewon fig tree seed shed, Bantul Yogyakarta, then carried out for taxonomic identification at the Biology Laboratory, Faculty of Biology, Ahmad Dahlan University. The testing animals were 3 mice obtained from the Yogyakarta White Rat Captivity, male Balb/c

*Corresponding author : Aji Winanta
Email : ajiwinanta@umy.ac.id

strains, 2-3 months old. This research has received ethical approval from the health research ethics committee, Faculty of Medicine and Health Sciences Universitas Muhammadiyah Yogyakarta.

Methods

Extraction

Fig *Simplicia* powder was extracted using a modified maceration method with remaceration. Fig powder 500 mg was put into a vessel and then added with 70% ethanol in a ratio of 1:6. It was left tightly closed for 5 days with daily stirring, then filtered using flannel and filter paper to separate the residue and the filtrate. The residue was then re-macerated for 5 days with a new liquid filter that was 3L of 70% ethanol. After the maceration and remaceration processes were completed, the macerate was then vaporized using a rotary evaporator at 100 rpm speed and a temperature of 60°C to obtain a thick extract.

Compound Identification Test by Thin Layer Chromatography (TLC)

Compound Identification by TLC refers to Anisa *et al.*'s (2018) procedure with some modifications. This test uses a stationary phase of silica gel GF254 and a mobile phase in the form of methanol: water with a ratio of 9:1. Rutin and quercetin used as standart. Then the extract and standard were elucidated in a TLC vessel containing the mobile phase. Elucidation was stopped when the mobile phase reached the limit. Then the spots on the plate were observed in visible light and UV254 nm and UV366 nm. Detection was carried out by spotting using ammonia vapor to detect flavonoids. Then the Rf value of the spots on the plate was measured.

Total Flavonoid Test

Using the colorimetric method, measurement of total flavonoid levels refers to Nurmila *et al.*'s procedure (2019), with some modifications with quercetin (QE) as the standard. The total flavonoid content of figs (*Ficus carica* L.) ethanol extract was measured with aluminum chloride (AlCl_3) reagent by visible spectrophotometry. A standard solution of 20 ppm quercetin or 0.5 mL of 40,000 sample solution was mixed with 1.5 mL of methanol, 0.1 mL of 10% AlCl_3 , 0.1 mL of 1 M Na. acetate, and ad aquades in a 5 mL volumetric flask and incubated for 30 minutes at room temperature (operating time results 30-50 minutes). The absorbance was then measured at a wavelength of 425 nm against the reagent blank consisting of methanol, AlCl_3 , and Na. Acetate and aquades. Each sample was replicated 3 times. Standard solutions of quercetin (QE) with

various concentrations of 2; 3; 4, 5, and 6 ppm in ethanol were used to construct the standard curves. The absorbance of the sample after deducting the absorbance of the blank sample (each sample solution without the addition of AlCl_3) was calculated for the total flavonoid content against the standard quercetin curve. Total flavonoid levels were expressed in mg quercetin equivalent per g sample (mg QE/g sample).

Macrophage Phagocytic Activity Test

Determination of macrophage phagocytic activity refers to the Munawaroh *et al.* (2018) procedure with several modifications. Macrophage cells were isolated from the peritoneal fluid of mice. Cells were counted with a hemocytometer, and then the pellet was resuspended with complete RPMI to produce a cell suspension of 2.5×10^6 cells/mL density. Cell suspensions were inoculated on 24-well plates covered with coverslips and incubated in a 5% CO_2 incubator at 37°C for 24 hours. Macrophage cells were washed with RPMI 1640 medium. Then 500 μL /well of the test material was added to both the sample concentration series (extract 62.5; 125; 250; 500 $\mu\text{g}/\text{mL}$), control cells, and replicated 3 times (3 coverslips). It was then incubated in a 5% CO_2 incubator at 37°C for 4 hours. The material was washed with RPMI-1640 medium, added 200 μL of $2.5 \times 10^6/\text{mL}$ latex suspension, and incubated for 1 hour. Cells were washed using PBS to remove residual latex and left to dry at room temperature, and fixed with methanol for 30 seconds. The methanol was dried, then the coverslips were stained with Giemsa 10% (v/v) for 20 minutes and dried at room temperature. The number of macrophages that phagocytize latex and the number of latex phagocytosed by active macrophages were then counted using an inverter microscope with 200x magnification until the number of macrophages observed is about 100.

$$\text{Phagocytosis index (PI):} \\ \frac{\text{Number of phagocytized latex}}{\text{Number of active macrophages (100)}}$$

$$\text{Phagocytosis Capacity:} \\ \frac{\text{The number of macrophages that phagocytize}}{\text{Number of Counted macrophages(100)}} \times 100\%$$

Lymphocyte Cell Proliferation Test

This study's determination of lymphocyte proliferation refers to the Khumaidi *et al.* (2020) procedure with some modifications. Isolation of lymphocyte cells was obtained from the extraction of the mice's spleen. The cell suspension was centrifuged, and the pellet was suspended in Tris Buffered Ammonium Chloride to lyse the

erythrocytes, then left at room temperature for 5 minutes. 5 mL of RPMI was added to obtain a suspension and then centrifuged at 2500 rpm for 4 minutes. The pellets were washed with RPMI, cells were resuspended with a complete RPMI medium, and cells were counted by a hemocytometer and an inverter microscope.

100 µL of lymphocyte cell suspension was added to a 96-multiwell plate with the addition of 10 µL of hepatitis B vaccine in each well. Then the plate was incubated in an incubator for 24 hours with a flow of 5% CO₂ at 37°C. Samples were then added in a concentration series of 62.5; 125; 250; 500 ppm for 3 replications and incubated for 48 hours. Then 10 µL of MTT was added to each well and incubated for 4 hours. 100 L of stopper reagent (10% SDS) in 0.01 N HCl was added to each well to stop the reaction. The absorbance was read with an ELISA reader at 550 nm then the proliferation of lymphocytes was counted using the stimulation index.

$$\text{Stimulation Index (SI)} = \frac{\text{Absorbance (Sample-medium)}}{\text{Absorbance (Normal-medium)}}$$

Statistical Analyses

Macrophage phagocyte index and lymphocyte proliferation were statistically analyzed using SPSS to determine whether there were significant differences between the treatment groups. The test used to determine the normality of the data distribution is Shapiro-wilk. If the data is normally distributed, then the analysis of variance is continued using Homogeneity of Variances. Data with identical variances can be continued with the One-Way Anova test with a 95% confidence level. It can be seen that there is no significant difference between the independent variables set. Then the data that are not normally distributed can be continued with the Kruskal-Wallis and Mann-Whitney test with a 95% confidence level.

RESULT AND DISCUSSION

Compound Identification Test by Thin Layer Chromatography (TLC)

In this study, analysis was carried out using TLC, which was used to separate the components present in the sample so that it could detect the chemical compounds contained in it. The stationary phase used in this research was silica gel GF254, and the mobile phase is methanol: water with a ratio of 9: 1.

The TLC test found that the fig extract contained flavonoid compounds characterized by yellow-brown spots after being evaporated by ammonia, which was visible in visible light, 254 nm

UV light, and yellow glow at 366 nm UV light. This color change was due to the reaction between flavonoid compounds and ammonia, which caused the conjugated double bond to become longer so that it could increase the color intensity of the spots. Compared to the comparisons, rutin, and quercetin, the spots from the ethanol extract of figs were suspected of containing flavonoids.

Total Flavonoid Test

Measurement of total flavonoid levels was carried out using the colorimetric method. The principle of this method is the formation of a complex compound between aluminum chloride with a keto group on the C-4 atom and a hydroxy group on the neighboring C-3 or C-5 atom from the flavone and flavonol groups (Supriningrum *et al.*, 2018). The maximum wavelength measurement was carried out in the 400-800nm range, and the obtained wavelength was 425 nm at a concentration of 20µg/ml; the maximum wavelength was then used to measure the absorption of the calibration curve and the ethanol extract sample of figs. Measuring the absorbance of the standard solution of quercetin at several concentrations (ppm) that were 2, 3, 4, 5, 6 obtained a linear relationship between concentration and absorbance of 0.9961, and the standard curve equation was $y = 0.0672x + 0.0896$. Based on linear regression, the total flavonoid content in fig extract was 0.74 ± 0.01 mg QE/gr sample. Oktavina Putri's research (2018) found that the total flavonoid content of fresh and dried fig leaves was $0.0105 \pm 0.003\%$ and $0.0025 \pm 0.0002\%$, respectively. The major component flavonoid of fig fruit is quercetin and luteolin, with 631 & 681 mg/Kg extract (Vaya and Mahmood, 2006).

Macrophage Phagocytic Activity Test

Macrophage phagocytosis is one of the non-specific immunological parameters in evaluating the immune system to see the cell's defense mechanism against foreign materials. Histologically, macrophages have relatively large cell nuclei and have an ameboid shape (the shape is not fixed). The plasma does not contain granules or agranulocytes. Macrophage cells have a small number and size is $\pm 9-12$ µm.

This macrophage phagocytic activity test was aimed to determine the effect of fig ethanol extract in increasing the activity of macrophage cells to phagocytize latex particles in vitro. Increased phagocytic index and phagocytic capacity of macrophages is a sign of increased phagocytic activity of macrophages. The ethanol extract of figs with the highest phagocytic index

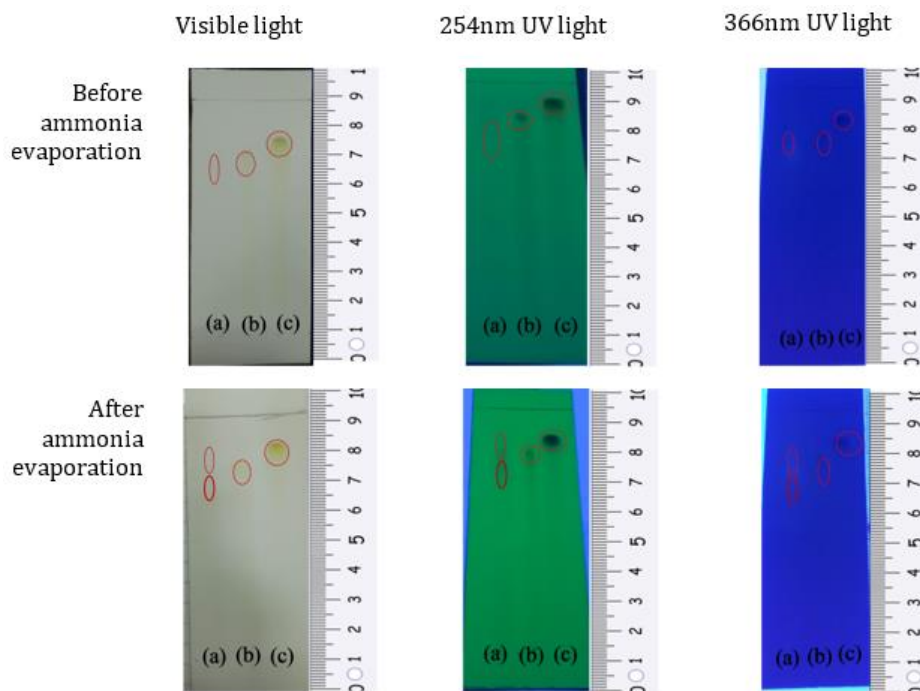


Figure 1. Results of TLC profile: (a) Fig fruit extract; (b) Rutin; (c) Quercetin, observation under visible light, UV254 and UV366; TLC system: stationary phase Silica Gel GF254, mobile phase methanol:water (9 : 1 v/v)

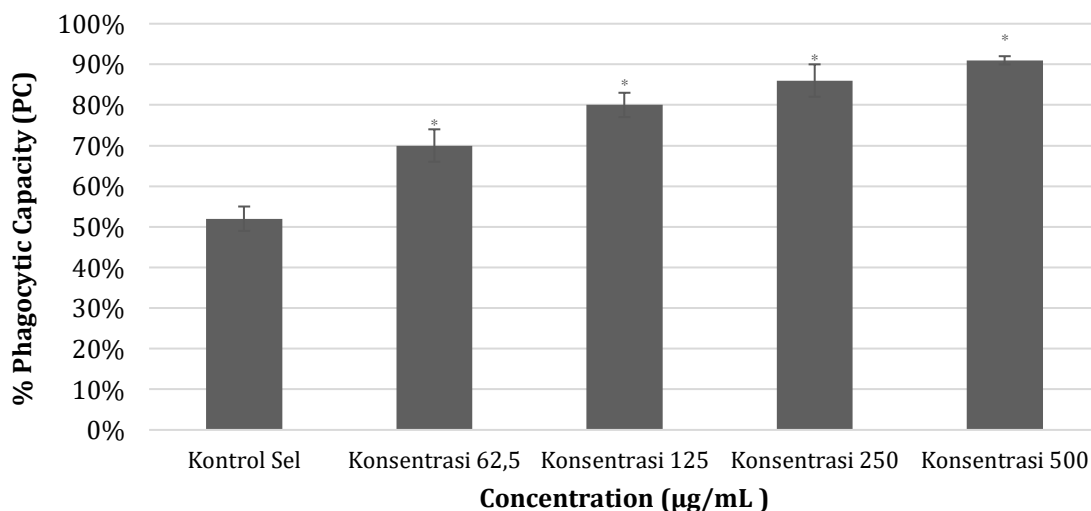


Figure 2. Profile of the phagocytic capacity of macrophages due to the administration of fig ethanol extract.

and phagocytic capacity was found at a 500 µg/mL (PI= 6.19 ± 0.31; % phagocytic capacity = 91 ± 0.01). Compounds that have a PI value > 1 are classified as immunostimulant compounds, which means the substance can stimulate or increase the body's resistance, while compounds with a PI value < 1 are classified as immunosuppressant compounds, namely substances that have the potential to suppress the immune system (Kresno,

2007). Therefore, based on Figure 3, fig extract has potential as an immunostimulant because it has a PI value >1.

In general, in the phagocytic activity of macrophages from the ethanol extract of figs produced when the higher the concentration given, the more flavonoid compounds there were. In the research of Munawaroh *et al.* (2018), it was shown that the ethyl acetate fraction of the ethanolic

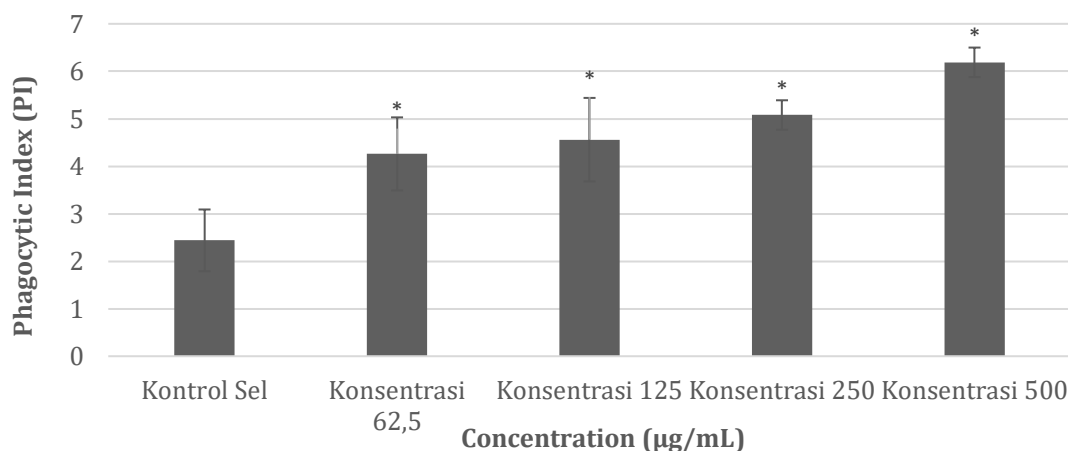


Figure 3. Profile of the phagocytic index of macrophages due to the administration of fig ethanol extract.

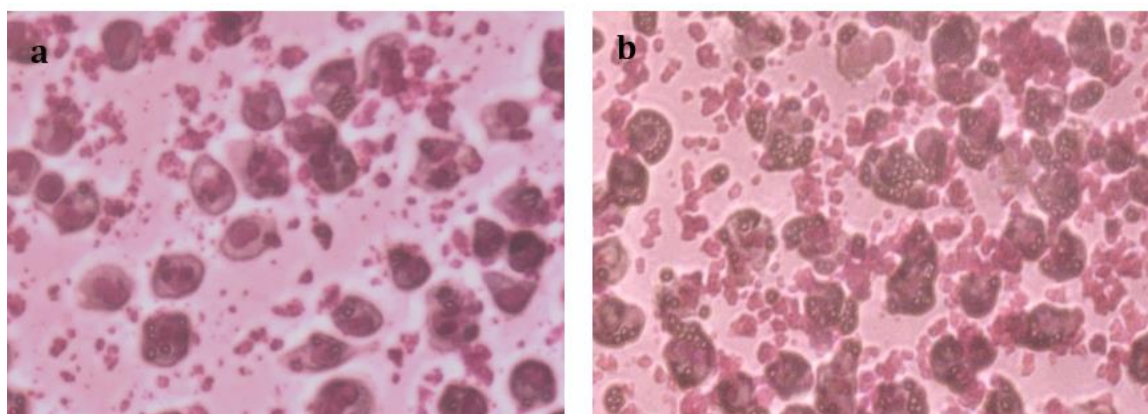


Figure 4. Comparison of the phagocytic activity of macrophage control cells (a) with macrophages after administration of fig extract (b), with a magnification of 200 times

extract of faloak (*Sterculia quadrifida* R.Br.) bark containing flavonoid compounds had macrophage phagocytic activity. Sulistiani and Rahayuning (2015) tested the effect of raw taro extract containing flavonoid compounds in increasing the phagocytosis index of macrophages. The increase in macrophage cell activity is due to the contained flavonoid compounds. Flavonoids work by activating NK cells to stimulate the production of IFN- γ . IFN- γ (Interferon- γ) will activate macrophages and stimulate increased phagocytic activity (Baratawidjaya, 2012). In Figure 4, macrophage cells that were not sampled had less cell activity in phagocytosing latex, but those sampled cells would have great activity in phagocytosing latex. This can be seen from the number of macrophages that actively phagocytize

and the amount of latex phagocytosed by macrophages.

Lymphocyte Cell Proliferation Test

Proliferation is a mitotic cell division cycle, where the parent DNA divides into two cells. Lymphocytes are a type of white blood cell (leukocyte). Lymphocytes are small round cells with a diameter of 7-12 μm , have a relatively large nucleus, round or slightly indented, surrounded by cytoplasm (Adinugroho *et al.*, 2019).

The immunomodulatory activity test of the sample against the lymphocyte proliferation test was carried out using the MTT Assay method.

Based on figure 5, the lymphocyte cell's proliferative activity in various concentrations shows that the administration of fig ethanol extract

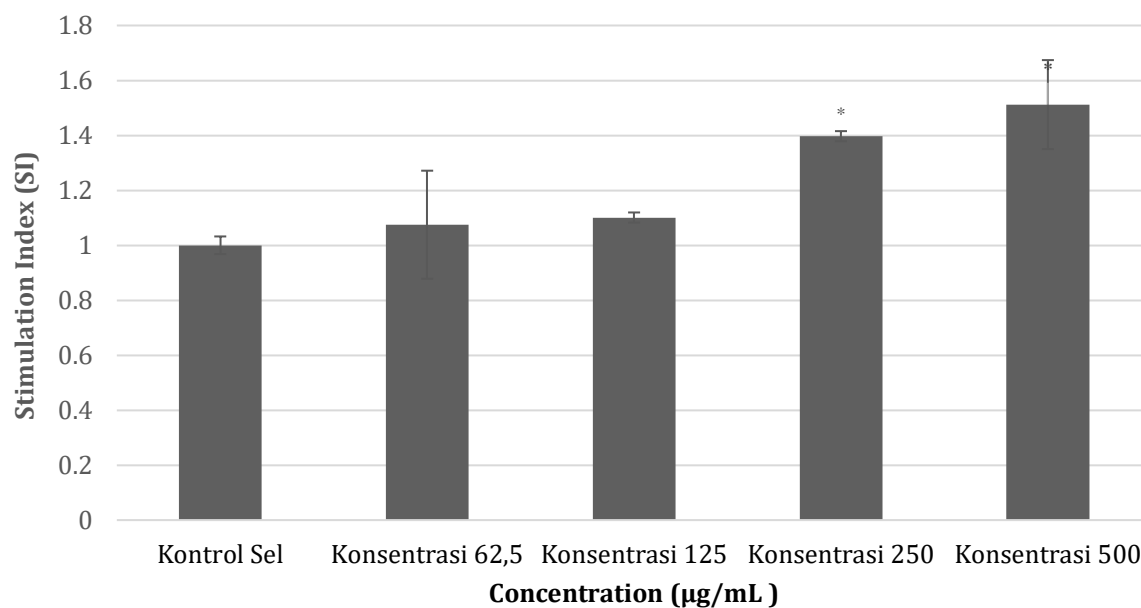


Figure 5. Profile of lymphocyte proliferation stimulant index due to the administration of fig ethanolic extract. Note: mean ± SD, n = 3, P < 0.05; * shows significant difference compared with control (P<0.05)

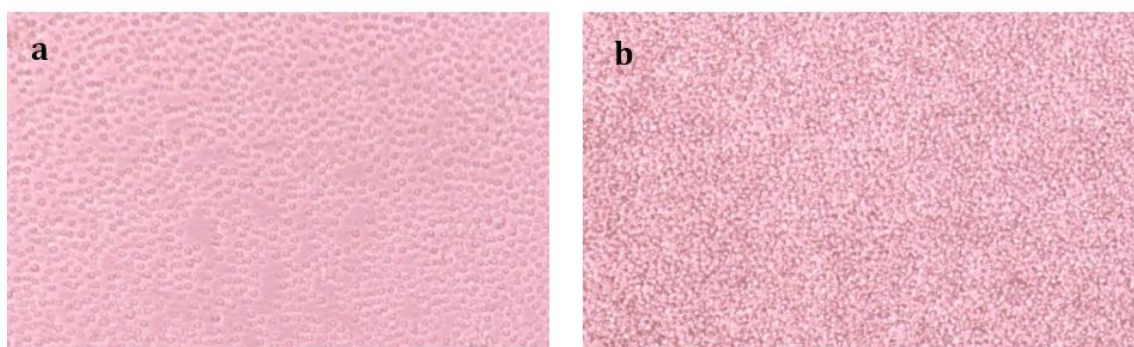


Figure 6. Comparison of lymphocyte cells in (a) control cells with (b) after administration of fig fruit ethanol extract

resulted in less than 2 proliferation index. These results indicated that the administration of extracts with those concentrations did not affect lymphocyte cell proliferation. The lymphocyte proliferation index of a compound generally interpreted to be SI between 2 and 3 is considered weakly positive and considered positive if SI > 3, primarily if positive results are obtained in more than one concentration (Hertiani *et al.*, 2019). In this study, lymphocyte cells were not very active in proliferating; this was possible because of gene suppression on lymphocyte cells or lack of nutrients in the media used. Cells need nutrients to get ATP energy needed in the cell proliferation process.

Data analysis based on the Shapiro-Wilk test on the stimulation index showed that the data were

normally distributed with a significant value > 0.05. Analysis of ANOVA variance with a 95% confidence level showed a significant value of <0.05, which means that the averages of various concentration variations were significantly different. However, at concentrations of 62.5 µg/mL and 125 µg/mL, there was no difference in the stimulation index (IS) of lymphocyte cell proliferation due to the administration of ethanol extract with control cells. At the concentrations of 250 µg/mL and 500 µg/mL, there were differences in the lymphocyte proliferation stimulation index.

The study by Khumaidi *et al.* (2020) showed that methanol extract, n-hexane fraction, ethyl acetate, and Begonia medicinal water increased lymphocyte cell proliferation (immunostimulator). The sample extract contains flavonoids that can

increase the production of IFN- γ but can reduce Th2 cytokines (IL-4) so that it can increase lymphocyte proliferation. Ulfah *et al.* (2018) showed that steeping soursop leaf tea has immunostimulatory activity against lymphocyte cell proliferation of Balb/C strain mice *in vitro*.

CONCLUSION

The ethanol extract of fig (*Ficus carica L.*) has a total flavonoid content value of the ethanol extract of fig (*Ficus carica L.*) is 0.74 ± 0.01 mgQE/gr sample. And has immunostimulant activity against macrophage phagocytic activity, which is significantly different from control cells but does not affect lymphocyte cell proliferation *in vitro*.

ACKNOWLEDGEMENT

The authors would like to thank the University of Muhammadiyah Yogyakarta which has provided research grants through the internal research program of lecturers for the 2020/2021 academic year and to the Cell Culture Laboratory of the Faculty of Medicine and Health Sciences which has assisted in this research.

REFERENCES

- Adinugroho, M. O., Suwiti, N. K., & Kendran, A. A. S. (2018). Histomorfometri sel darah putih agranulosit bibit sapi bali di nusa penida. *Buletin Veteriner Udayana*, 33-38.
- Anisa, K., Rahayu, T., & Hayati, A. (2018). Profil metabolit sekunder daun tin (*Ficus carica*) melalui analisis histokimia dan deteksi flavonoid dengan metode kromatografi lapis tipis (klt). *Jurnal SAINS ALAMI (Known Nature)*, 1(1).
- Baratawidjaja, K.G. dan Rengganis, I., 2012, *Imunologi Dasar*, Edisi ke-10, Balai Penerbit Fakultas Kedokteran Universitas Indonesia, Jakarta. 29-32, 61-64, 69, 73, 583
- Carmelita, A. B. C. B. (2016). Pengaruh Pemberian Ekstrak Etanol Umbi Bawang Dayak (*Eleutherine palmifolia* (L.) Merr.) Secara Oral pada Mencit BALB/c Terhadap Pencegahan Penurunan Diameter Germinal Center pada Kelenjar Getah Bening Serta Kadar IgG Serum. *Jurnal Biosains Pascasarjana*, 18(1).
- Hertiani, Triana, *et al.*, (2019), *In Vitro Immunomodulatory and Cytotoxic Potentials of Faloak (Sterculia quadrifida R.Br.) Bark*, *OnLine Journal of Biological Sciences* Volume 19 No. 4, 222-231
- Jumiarni, W. O., & Komalasari, O. (2017). Inventory of Medicines Plant as Utilized by Muna Tribe in Kota Wuna Settlement. *Majalah Obat Tradisional*, 22(1), 45-56.
- Khumaidi, A., Widodo, A., Nugrahani, A. W., Sasmito, E., & Fakhrudin, N. (2020). Lymphocyte Cell Proliferation Profile of Benalu Batu (*Begonia medicinalis*) from the North Morowali Regency Central Sulawesi Province. *JURNAL ILMU KEFARMASIAN INDONESIA*, 18(1), 61-67.
- Krensky, A., Vincenti, F., and Bennet, W., (2006), *'Immunosuppressants, Tolerogens, and Immunostimulant'*, in: *Goodman & Gilman's the Pharmacological Basis of Therapeutics*, 11th Edition, McGraw-Hill, New York.
- Kresno, S.B. (2007). *Imunologi: Diagnosis dan Prosedur Laboratorium. Edisi IV*, Cetakan ke 83. Jakarta: Penerbit Universitas Indonesia
- Munawaroh, R., et al. (2018). Korelasi Antara Kadar Flavonoid Total dan Aktivitas Fagositosis Makrofag Fraksi-Fraksi dari Ekstrak Etanol Kulit Batang Faloak (*Sterculia quadrifida* R. Br.) Secara *In Vitro*. *Traditional Medicine Journal*, Vol. 23(1), p 47-55.
- Nurmila, N., Sinay, H., & Watuguly, T. (2019). Identifikasi dan Analisis Kadar Flavonoid Ekstrak Getah Angsana (*Pterocarpus indicus* Willd) Di Dusun Wanath Kecamatan Leihitu Kabupaten Maluku Tengah. *BIOPENDIX: Jurnal Biologi, Pendidikan Dan Terapan*, 5(2), 65-71.
- Putri, O. K. (2018). Kadar Fenolik Total dan Flavonoid Total Seduhan Daun Tin (*Ficus carica*) Segar dan Kering dengan Air Mendidih. *JC-T (Journal Cis-Trans): Jurnal Kimia dan Terapannya*, 2(2).
- Sulistiani, R. P., & Rahayuningsih, H. M. (2015). Pengaruh Ekstrak Lompong Mentah (*Colocasia Esculenta* L Schoot) Terhadap Aktivitas Fagositosis Dan Kadar NO (Nitrit Oksida) Mencit Balb/C Sebelum Dan Sesudah Terinfeksi *Listeria Monocytogenes*. *Journal of Nutrition College*, 4(4), 409-415.
- Supriningrum, R., Sundu, R., & Setyawati, D. (2018). Penetapan Kadar Flavonoid Ekstrak Daun singkil (*Premna corymbosa*) Berdasarkan Variasi Suhu dan Waktu Pengeringan Simplisia. *JFL: Jurnal Farmasi Lampung*.
- Ulfah, M., Cahyani, V. S. N., & Kinasih, I. (2018). Pengaruh pemberian seduhan teh daun sirsak (*Annona muricata* L.) terhadap aktivitas fagositosis sel makrofag dan proliferasi sel limfosit mencit galur BALB/c yang diinduksi vaksin hepatitis B. *Jurnal Ilmiah MOMENTUM*, 13(2).
- Vaya, J, and Mahmood, S. (2006). Flavonoid Content in leaf extracts of fig (*Ficus carica*

Iis Nur Azizah

L.), carob (*Ceratonia siliqua* L.) and pistachio (*Pistacia lentiscus* L.). *BioFactors*, 28, 169-175.
Widyaningrum, N., Hussana, A., Adi, R. S., Tiastuti,

M., & Utami, K. M. (2020). Cytotoxic Activity Of Combined Fig Extract And Olive Oil Against Breast Cancer Cells. *Jurnal Farmasi Sains dan Praktis*, 6(1), 1-8.