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Original Article

Immunostimulating Study of Active Agent Fraction from Sungkai (*Peronema canescens* Jack.) Leaf from SARS-COV-2 Virus Antigen Exposure to NK and CD8+T Cells

Dwisari Dillasamola[1,*], Fatma Sri Wahyuni[1], Rauza Sukma Rita[2], Dachriyanus[1], Yohanes Alen[1], Salman Umar[1], Yufri Aldi[1]

ABSTRACT

Introduction: Sungkai (*Peronema canescens* Jack.) plant had been used as an immune system enhancer. **Aim**: In this study, the effect of Sungkai leaf extracts from 4 different fractions, namely n-hexane, ethyl acetate, butanol and residual water with 3 variations in doses of 1,10 and 100 mg/kg bw on the activity of NK and CD8+T cells in male white mice that have been exposed to SARS-Cov-2 virus antigen was investigated. **Methods:** The experimental animals used were 60 animals divided into 12 groups with 14 days of treatment which had previously been induced with SARS-Cov-2 virus antigen (Moderna) and given with Sungkai leaf extracts for 14 days and evaluated on day 15. The evaluation results of NK cells concentrations sequentially were 2.96; 4.66; 5.38; 5.43; 4.05; 2.89; 3.56; 4.21; 2.88; 1.99; 2.07; 4.40; 3.21; 3.40; and 6.93 ng/ml. On the other hand, the evaluation results of CD8+T cells concentrations sequentially were 27.47; 28.96; 29.19; 27.90; 21.85; 25.79; 27.98; 23.50; 23.39; 26.56; 22.62; 25.19; 23,55; 26,75; and 29,69 ng/ml. One-way ANOVA and Duncan test were used for the data analysis. **Results:** The results showed significant increase of concentration ($p<0.05$) towards concentration of NK cells in the butanol fraction at a dose of 1 mg/kg BW and CD8+T cells in the residual water fraction at a dose of 100 mg/kg BW. **Conclusion:** It can be concluded that fraction from sungkai (*Peronema canescens* Jack.) at doses of 1,10 and 100 mg/kg bw shows immunostimulatory activity.

Key words: *Peronema canescens* Jack., NK Cells, CD8+T Cells, SARS-Cov-2.

Dwisari Dillasamola[1,*], Fatma Sri Wahyuni[1], Rauza Sukma Rita[2], Dachriyanus[1], Yohanes Alen[1], Salman Umar[1], Yufri Aldi[1]

[1]Faculty of Pharmacy Universitas, Andalas, INDONESIA.

[2]Faculty of Medicine Universitas, Andalas, INDONESIA.

Correspondence

Dwisari Dillasamola

Faculty of Pharmacy Universitas, Andalas, INDONESIA.

E-mail: dwisaridillasamola@phar.unand.ac.id

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INTRODUCTION

The immune system or immune system is the body's defense mechanism that functions to respond to or respond to "attacks" from outside the body. When an attack occurs, the antigen will begin to stimulate the immune system. This mechanism will protect the body from attacks by various microorganisms such as bacteria, viruses, fungi, and various germs that cause disease. When the immune system does not work optimally, the body will be vulnerable to disease. Some things that affect the immune system for example food, environmental factors, daily lifestyle, age, stress, and hormones. Therefore, everyone is advised to maintain a healthy and good lifestyle with a healthy way of life, namely consuming healthy foods with balanced nutrition and regular exercise.[1]

An immunostimulant can increase body defense mechanism. Generally, an immunostimulant is defined as a compound that can increase body defense mechanism specifcally and nonspecifically through cellular or humoral response. Certain plants contains compound that show immunostimulatory activity.[2] The components of the body's immune system that are very important in protecting the body against the Covid-19 virus attack are Natural Killer (NK) and CD8+ T cells. These two cells protect the body against Covid-19 virus infection by producing a group of cytokines in the form of interferon, granzyme, and perforin compounds as well as pro-inflammatory cytokines known as cytokine storms.

The immune system can be divided into two types; the non-specific immune system (innate immunity) and the specific immune system (adaptive immunity). The non-specific immune system has a faster activity because it does not involve the memory cells.[3] One of components that are involved in the non-specific immune system is natural killer cells which protect the body from pathogen attacks so that the body eventually builds its defense system. The body's defense system can be activated by providing compounds that can increase the body's imune response.[4]

Body protection against viral exposure is through the activity of NK cells, where NK cells will release interferon, perforin, and granzyme compounds. Interferon play a role in inhibiting the proliferation and differentiation of viruses, besides that they can also inhibit the attachment of the virus to receptors in normal cells, so that normal cells are not infected, perforin compounds play a role in disrupting permeability or leakage from the cytoplasmic membrane of cells infected with the virus so that the cells die. Granzyme compounds function in the process of apoptosis for cells experiencing viral infection so that the cells die.[2]

CD8+T cells are a type of white blood cell that help regulate and carry out the body's immune response. CD8+T cells include suppressor T cells and cytotoxic T lymphocytes or T-8 cells (CD8+). In the regulation of cytokines in the immune response, CD8+ cells kill infected target cells by releasing lytic granules (perforin and granzyme) or by induction of production (FasL) or TNF-alpha, which by binding to their receptors initiates cell apoptosis. target.

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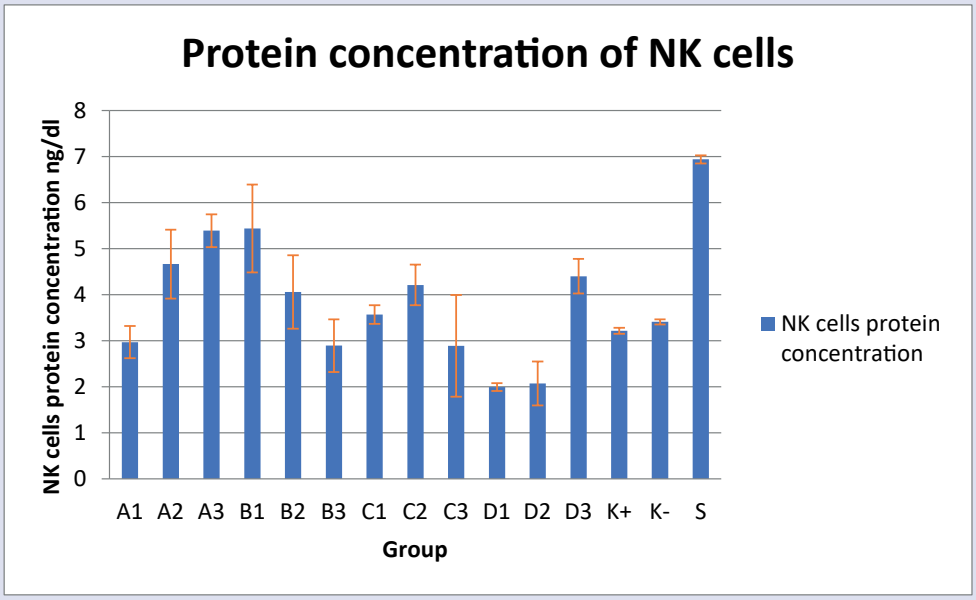

Figure 1: Protein concentrations of NK cells.

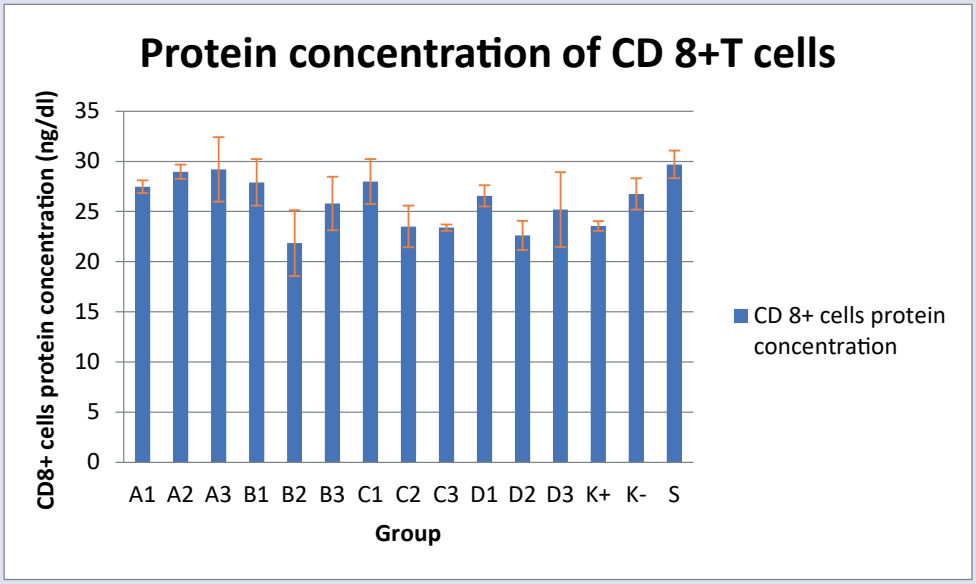

Figure 2: Protein concentrations of CD8+T cells.

Some of the plants used by the community to increase endurance used by the community are Sungkai (*Peronema canescens* Jack.). All these plants are abundant in West Sumatra. This medicinal plant has properties as an immunomodulatory agent or immune system enhancer. In the study[5] that the dose of *Peronema canesens* Jack. for the antibacterial species of the protozoan parasite Babesia gibsoni, the dose was 0.7 g/kg BW and did not cause toxicity in the mice tested and in the study,[6] *Peronema canesens* Jack. a dose of 0.5625 mg/kg BW can increase the number of leukocytes by 36%. Ningsih's research results in 2013 show that the ethanol extract from the leaves of *Peronema canescens* Jack. has antiplasmodial activity *in vivo* with its ED50 value of 102.88 mg/kg BW. Several research findings of Sungkai plants have secondary metabolites in their leaves. The method of obtaining these compounds is by extracting Sungkai leaves using a solvent. The findings of secondary metabolites contained in the form of flavonoid, phenolic, and terpenoid compounds. The scientific process is very important so that the use of traditional medicine is not based on experience alone but has scientific evidence so that it can be used in a modern formal health care system.[6] Based on the description above, researchers are interested in researching the activity of standardized extracts from the leaves of the Sungkai *Peronema canesens* Jack. protection against SARS-CoV-2 virus infection by observing the activity of NK and CD8+ T cells.

Based on the description above, researchers are interested in researching the activity of standardized extracts from the leaves of the Sungkai *Peronema canesens* Jack. with several variations of the solvent fraction against the protection of SARS-CoV-2 virus infection by observing cell activity in the form of NK cells and CD8+T cell activity.

METHODS AND MATERIALS

Time and place research

The research was conducted from June 2021 to June 2022 at the Cell Culture Laboratory, Pharmacology Laboratory, Immunology, and

Serology Laboratory, and the Central Laboratory of the Faculty of Pharmacy, Universitas Andalas.

Tools

The tools used were Evaporator (Buchi R-210 Rotavapor), UV-vis spectrophotometer (Thermo Scientific Genesys 10S UV-Vis), UV-lamp (Camag), beaker glass (Pyrex), measuring cup (Pyrex), probe needle (Terumo), volumetric flask (Pyrex), volumetric pipette (Pyrex), digital analytical balance, Silica gel 60 F254 (Merck), desiccator, spatel, dark bottle, TLC plate (Merck), Erlenmeyer (Pyrex), centrifuge (Oregon) , incubator (Gallenkamp plus), mortar, probe, surgical instrument, filter paper, animal cage, oven, haemocytometer, BIO-RAD spectrophotometer, crucible, test tube, dropper, microscope (Olympus), gloves (Sensi) , mask (Sensi), oven (Carbolite Gero®), grinder, stemper mortar, test tube, porcelain exchanger, hot plate®), rubber gloves, spray bottle, T-25 flask (Iwaki®), micro pipette (Ecopipette®), hemacytometer, analytical balance (Mettler Toledo®), refrigerator (Samsung®), Incubator 37ºC/5%CO2 (Thermo Scientific®), microbiological safety cabinet air flow class II (Therm o Scientific®), water bath (Memert®), microplate reader (Bio-Rad®), centrifuge (Thermo Scientific®), conickel tube (Falcon®), eppendorf tube, inverted microscope (Zeiss®), 96 plates and 24 wells , plastic bags, tissue, and stationery.

Material

The materials used were Sungkai extract (*Peronema canesens Jack.*), aquadest, 0.5% sodium CMC, ethanol P, physiological NaCl 0.9% NaCl,

Table 1: Extract yield of Sungkai leaf.

Sample Weight (g)	Extract Weight (g)	% Yield
2.400	387.5	16.14%

Table 2: Organoleptic examination of Sungkai leaf extract fraction.

Examination	Fraction Observation			
	N-hexane	Ethyl Acetate	Butanol	Water
Shape	Glutinous	Glutinous	Glutinous	Thick
Color	Blackish brownish green	Blackish brownish green	Blackish brownish green	Blackish brown
Odor	Peculiar	Peculiar	Peculiar	Peculiar
Taste	Bitter	Bitter	Bitter	Bitter

Table 3: Phytochemical screening of Sungkai leaf extract fraction.

Examination	Fraction Observation			
	N-hexane	Ethyl Acetate	Butanol	Water
Flavonoids	+	+	+	+
Alkaloids	-	+	+	-
Saponins	+	+	+	+
Phenolics	+	+	+	+
Steroids/Terpenoids	-/+	-/+	-/+	+/-

Table 4: Protein concentrations of NK cells.

Groups	Protein concentrations of NK cells					Mean	SD
	I	II	III	IV	V		
A1	2.431	3.367	2.899	3.133	3.016	2.969	0.347
A2	3.367	4.694	5.215	4.954	5.84	4.663	0.749
A3	5.839	5.267	4.876	5.553	5.410	5.389	0.356
B1	3.758	5.579	6.073	5.826	5.949	**5.437**	0.956
B2	4.564	4.928	3.601	2.925	4.264	4.056	0.798
B3	3.185	3.653	2.170	2.911	2.540	2.892	0.572
C1	3.784	3.263	3.706	3.484	3.595	3.566	0.203
C2	4.694	4.616	3.680	4.148	3.914	4.210	0.439
C3	4.824	2.743	2.144	2.443	2.293	2.889	1.103
D1	1.858	2.092	1.975	2.033	2.004	1.992	0.086
D2	2.300	1.312	2.561	1.936	2.248	2.071	0.479
D3	4.954	3.940	4.512	4.226	4.369	4.400	0.374
K+	3.148	3.172	3.339	3.160	3.249	3.213	0.065
K-	3.306	3.482	3.442	3.394	3.418	3.408	0.053
S	6.931	6.809	7.095	6.870	6.982	6.937	0.089

Description:
A : Water fraction
B : Butanol fraction C : N-hexane fraction D : Ethyl acetate fraction K : Control S : Stimuno
1 : Dose of 1 mg/kgbw
2 : Dose of 10 mg/kgbw
: Dose of 100 mg/kgbw

Table 5: Protein concentrations of CD8+ cells.

Protein concentrations of CD8+T cells							
Groups	I	II	III	IV	V	Mean	SD
A1	26.901	28.310	26.824	27.567	27.195	27.474	0.634
A2	28.458	28.087	29.867	28.977	29.422	28.962	0.715
A3	24.677	27.272	32.696	29.984	31.340	**29.193**	3.224
B1	23.936	29.941	28.087	29.014	28.550	27.905	2.322
B2	18.302	21.119	21.341	27.272	21.230	21.852	3.284
B3	21.045	26.605	27.272	269.385	27.105	25.793	2.665
C1	26.901	24.529	29.496	29.496	29.496	27.983	2.234
C2	27.124	23.269	22.083	22.676	22.379	23.506	2.069
C3	22.898	23.269	23.714	23.491	23.602	23.395	0.322
D1	24.900	27.791	26.345	27.068	26.706	26.562	1.072
D2	25.122	21.416	22.453	21.934	22.193	22.623	1.448
D3	26.012	18.599	27.494	26.753	27.123	25.196	3.728
K+	23,310	23,020	24,470	23,160	23,820	23,556	0,484
K-	23,780	28,430	28,260	26,100	27,180	26,750	1,556
S	29,300	31,980	27,500	30,640	29,070	29,698	1,383

Description:
A : Water fraction
B : Butanol fraction C : N-hexane fraction D : Ethyl acetate fraction K : Control S : Stimuno
1 : Dose of 1 mg/kgbw
2 : Dose of 10 mg/kgbw
3 : Dose of 100 mg/kgbw

ethanol 70%, silica gel TLC plate F254, aquadest, F254, Mayer reagent, Dragendorf reagent, HCl, Mg metal, FeCl3, HgCl2, AlCl3, sodium acetate, Mouse NK and CD8+T Bt laboratory kit cells, Moderna Vaccine, white male mice, and Wright stain, EDTA.

Sungkai leaf extraction and fractionation

Sungkai leaf powder (*Peronema canesens* Jack.) was extracted by multistage maceration method, to obtain non-polar, semi-polar, and polar fractions. Maceration was started by using a non-polar solvent n-hexane with a ratio of 1:10, maceration was carried out according to the standard method in the pharmacopeia. After the maceration solvent obtained was clear, the maceration was continued by using semi-polar ethyl acetate solvent in a ratio of 1:10, after the maceration solvent with ethyl acetate, was obtained clear, the maceration was continued by using butanol solvent in a ratio of 1:10. So that obtained fractions of n-hexane, ethyl acetate, butanol, and residual water. Then the solvent for each fraction was evaporated using a rotary evaporator to obtain a thick fraction.[7,8]

Preparation of experimental animal

The animals used were 60 male white mice aged 2-3 months with a bodyweight of 20-30 grams and had never been treated with drugs. Before being used as experimental animals, mice were adapted for 7 days to adjust to the environment and control health and body weight as well as uniform food. These experimental animals were grouped into 12 groups consisting of 5 mice in one group for 1 extract in 4 fractions and 3 dose variations. Mice were given the Sungkai leaf extract fraction for 14 days and on day 0 were given the Moderna vaccine blood was taken and serum was taken which was then checked for protein concentrations of NK and CD8+ T cells using the ELISA technique. The principle of the sandwich ELISA method is the occurrence of a complex bond between the antigen and 2 antibodies, the first antibody (capture antibody) is coated into the well on the microplate and the second antibody is conjugated with an enzyme (detection antibody). The addition of the substrate will cause a color change which indicates the presence of antigen in the sample. Absorption measurements were carried out tosdetermine the amount of antigen in the sample.[9]

Dosage determination

The dose of Sungkai leaf extract used in this study was given in 3 dosage variants, namely 1, 10, and 100 mg/k bw.[10]

NK and CD8+T Cell Count

On the eighth day, blood was drawn by guillotine (neck artery). Then the blood was collected and then centrifuged for 30 minutes at 3000 rpm to obtain serum. Then the serum was used to test the levels of NK cells and CD8+T cells by the ELISA method.[11]

Data analysis

The data obtained from the research results were analyzed statistically using the one-way Analysis of Variance (ANOVA) method and continued with Duncan's analysis using SPSS statistical software.[12]

RESULT

Extract yield

a. Extract yield of Sungkai leaf

b. Organoleptic examination of Sungkai leaf extract fraction

c. Phytochemical screening of Sungkai leaf extract fraction

d. Protein concentrations of NK cells and CD8+ T cells

DISCUSSION/CONCLUSION

Plant identification was carried out at the Laboratory Herbarium with indentfication number 145/K-ID/ANDA/III/2022, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas Limau Manih Campus, Padang, West Sumatra. Based on the results of plant identification used in this study are explained as the following;

The process of obtaining Sungkai leaf extract (*Peronema canescens* Jack.) begins with collecting fresh Sungkai leaves, cleaning them from impurities, washed with running water, and then air-dried to become dry Simplicia, then after the dried Simplicia is mashed using a blender and obtained Simplicia powder, maceration sample is carried out by

using 70% ethanol solvent.[8] From 2400 grams of Simplicia powder, 387.5 grams of thick extract were obtained and the yield value of the extract was 16.14%. Sungkai leaf powder (*Peronema canesens* Jack) was extracted by the graded maceration method, to obtain non-polar, semi-polar, and polar fractions. Maceration was started by using a non-polar solvent n-hexane with a ratio of 1:10 where it was carried out according to the standard method in the pharmacopeia. After the solvent obtained from maceration was clear, the maceration was continued using semi-polar solvent ethyl acetate with a ratio of 1:10. After the solvent obtained from maceration with ethyl acetate was clear, the maceration was continued by using butanol solvent in a ratio of 1:10 to obtain the n-hexane fraction, ethyl acetate, butanol, and residual water. The solvent for each fraction was evaporated using a rotary evaporator to obtain a viscous fraction. Then, standardization of Sungkai leaf (*Peronema canescens* Jack.) extract drying shrinkage obtained was 8.91% which complies with the provisions of the Indonesian Herbal Pharmacopoeia where the drying shrinkage of the Gotu Kola herb extract should be not more than 10%. The determination results of the total ash content of Sungkai leaf (*Peronema canescens* Jack.) extract is 3.66% which also meets the provisions of the Indonesian Herbal Pharmacopeia where the total ash content of the thick extract of Sungkai leaf should be not more than 10%.

The organoleptic examination was aimed at a simple initial identification of the Sungkai leaf extract used, namely in the form of a viscous fraction having a characteristic odor, blackish-brown color, and bitter taste. The chemical content test of the extract included a phytochemical test, which showed that the condensed fraction of Sungkai leaves was positive for flavonoids, phenolics, saponins, and terpenoids.

On the Thin Layer Chromatography of the extract, the eluent or mobile phase used was Ethyl acetate: methanol (2:3), and using a silica gel plate F254 as the stationary phase, the comparison used was quercetin and obtained an Rf value of 0.54 cm.

Based on the results of the test using the ELISA method, the increase in NK cells was found in group B1 which was 5,437 ng/ml, namely the butanol fraction at a dose of 1 mg/kgbw with treatment from days 1-7 given the Sungkai leaf extract fraction. Based on the one-way ANOVA statistical test, showed that there were significant differences between the 12 treatment groups (P-value < 0.05). The results of the calculation of the average protein concentration of NK cells in male white mice given several fractions of Sungkai leaf extract and exposed to Sars-Cov-2 virus antigen sequentially from group 1 to group 12 were 2.96; 4.66; 5.38; 5.43; 4.05; 2.89; 3.56; 4.21; 2.88; 1.99; 2.07; 4.40; 3.21; 3.40; and 6.93 ng/ml. NK cells play an important role in nonspecific immunity against intracellular pathogens that can recognize and kill abnormal cells, and destroy cells that contain viruses or neoplasm cells. NK cells are activated by interferons which are normally produced and released by virus-infected cells themselves. Interferons affect accelerating maturation and cytolytic effects of NK cells. Interferon also causes increased resistance to viruses in uninfected cells.[13] NK cells are a subset of lymphocytes with either a CD 16 or CD 56 surface (a receptor for FC). NK cells are a subset of lymphocytes with either a CD 16 or CD 56 surface (a receptor for FC). Surface features of CD 16 and CD 56 have been used to date to ensure that these cells are NK cells that can differentiate between T cells and B cells. it also does not interact with the T cell receptor (TCR).[14] NK cells play an important role in the natural defense against the growth of cancer cells and various infectious diseases, especially viral infections. Most of the NK cells (95%) can function as killer cells, virus-infected cells, and other target cells that are coated with immunoglobulin G (IgG) so that they function as antibody-dependent cell-mediated cytotoxicity or ADCC.[14] NK cells are components of innate immunity that act as killers (cytotoxicity) by secreting lysosomes containing perforins and granzymes and also producing cytokines IFN-γ, TNF-α, IL-5, IL-13.[15] NK cells also function as co-stimulatory that can stimulate macrophages, T cells, and B cells, thus bridging the interaction between innate immunity and adaptive immunity.[16]

The results of the protein concentration calculation of male white mouse CD8 + T cells after administration of the highest Sungkai leaf extract fraction was in group A3, which was 29,193 ng/ml in the water fraction with a dose of 100 mg with the extract from days 1-7. Based on the one-way ANOVA statistical test, showed that there were significant differences between the 12 treatment groups (P-value <0.05). The results of the calculation of the average concentration of male white mouse CD8+T cells given several fractions of Sungkai leaf extract and exposed to Sars-Cov-2 virus antigen sequentially from group 1 to group 12 were 27.47; 28.96; 29.19; 27.90; 21.85; 25.79; 27.98; 23.50; 23.39; 26.56; 22.62; 25.19; 23,55; 26,75; and 29,69 ng/ml. CD8+T cells express the CD8+T cells coreceptor and destroy infected cells between dependent MHC-I specific antigens. CD8+T cells can kill cells directly and through the induction of apoptosis.[17] CD8+T cells contain abundant azurophilic granules and are capable of destroying various tumor cells, infected cells, and abnormal cells without prior sensitization.[18] An effective viral response from the host is carried out by the innate and adaptive immune systems through the production of various proinflammatory cytokines, and activation of T cells, CD 4, and CD8+T cells. T cells are important for controlling viral replication, limiting virus spread, and clearing infected cells. However, tissue caused by viruses can lead to overproduction of proinflammatory cytokines, and recruitment of proinflammatory macrophages and granulocytes. This situation is known as a cytokine storm which can lead to more severe tissue damage.[19]

SUMMARY

Sungkai leaf plant had been used as an immune system enhancer. In this study, the effect of Sungkai leaf extracts from 4 different fractions, namely n-hexane, ethyl acetate, butanol, and residual water, with 3 variations in doses of 1, 10, and 100 mg/k bw on the activity of NK and CD8+T cells in male white mice that have been exposed to SARS-Cov-2 virus antigen was investigated. The experimental animals used were 60 animals divided into 12 groups with 14 days of treatment which had previously been induced with SARS-Cov-2 virus antigen (moderna) and given with Sungkai leaf extracts for 14 days and evaluated on day 8. The evaluation results of NK cell concentrations sequentially were 2.96; 4.66; 5.38; 5.43; 4.05; 2.89; 3.56; 4.21; 2.88; 1.99; 2.07; 4.40; 3.21; 3.40; and 6.93 ng/ml. On the other hand, the evaluation results of CD8+T cells concentrations sequentially were 27.47; 28.96; 29.19; 27.90; 21.85; 25.79; 27.98; 23.50; 23.39; 26.56; 22.62; 25.19; 23,55; 26,75; and 29,69 ng/ml. One-way ANOVA and Duncan test were used for the data analysis. The results showed that there was an increase in the concentration of NK cells in the butanol fraction at a dose of 1 mg/kg BW and CD8+T cells in the aqueous fraction at a dose of 100 mg/kg BW.

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CONFLICTS OF INTEREST

There is no conflicts of interest in this study.

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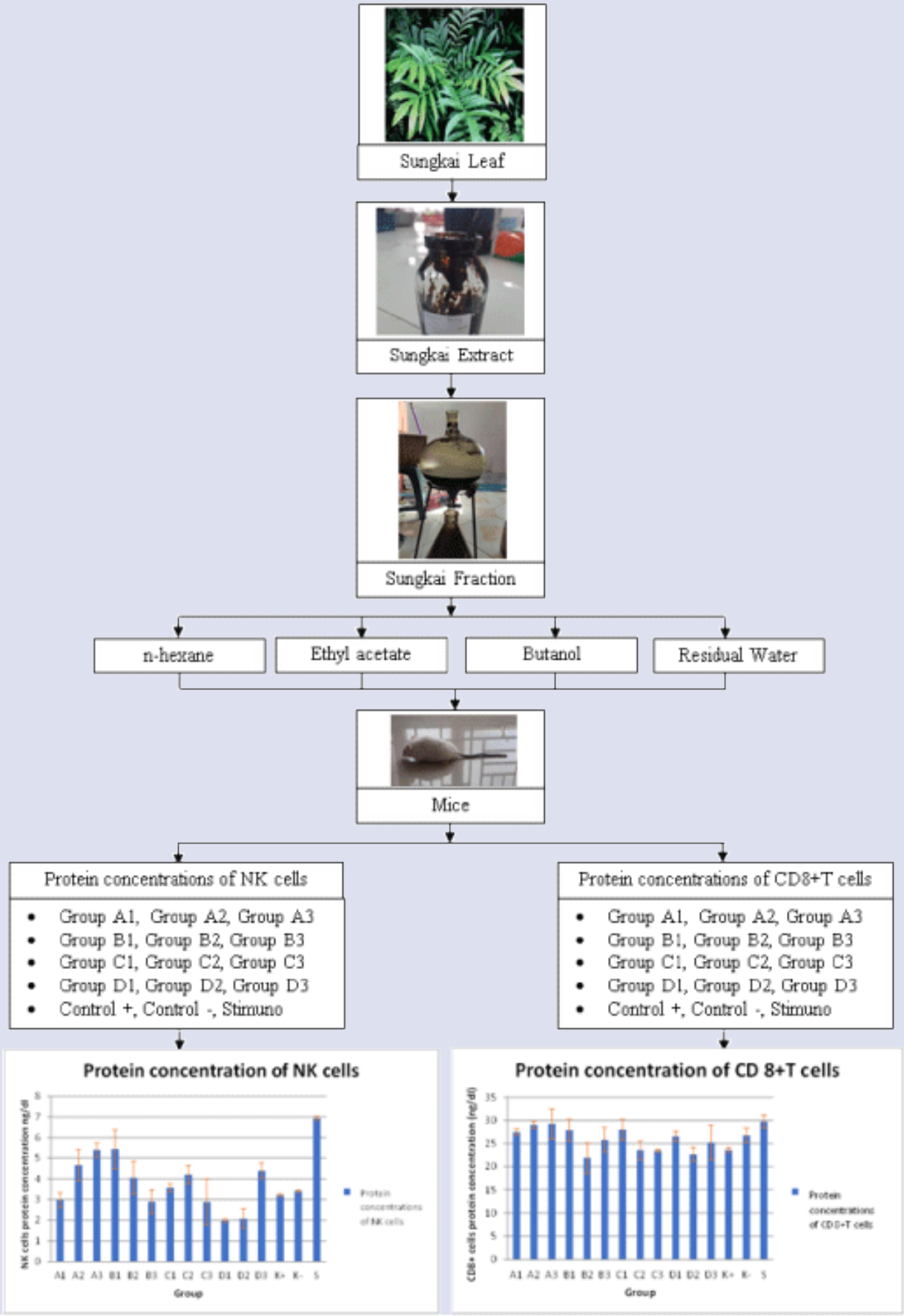
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Dwisari dillasamola currently as a lecturer functional positions at the Faculty of Pharmacy, Universitas Andalas. Graduated from Faculty of Pharmacy Universitas Andalas in 2004, then Master Program at Faculty of Pharmacy Universitas Andalas in 2011. The research and expertise are in Farmaco-Immunology. Currently working as lecturer of Farmaco-Immunology of Faculty of Pharmacy, Universitas Andalas.

Fatma Sri Wahyuni is a lecture at the Department of Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatera, Indonesia. Got her undergraduate degrees from Universitas Andalas in 1998 and finished her PhD from University Putra Malaysia in 2010. Her research interest is in the area of cytotoxic study of Sumatran medicinal plants especially genus of Garcinia.

Rauza Sukma Rita is a lecturer at the Department of Biochemistry, Faculty of Medicine, Universitas Andalas, Padang, Indonesia. In 2009, she received her Medical Doctor from the Faculty of Medicine, Universitas Andalas. She completed her education at Jichi Medical University in Japan, where she received her Ph.D. in 2015. Her areas of interest in the study include diabetes, obesity, oxidative stress, other degenerative disorders, and herbal medicine.

Dachriyanus currently as a lecturer functional positions at the Faculty of Pharmacy, Universitas Andalas**.** Graduated from Universitas Andalas in 1991 and completed his PhD from The University Of Western Australia in 2000. His research interest is in drug discovery. Currently working as a lecturer at the Department of Pharmacy, Faculty of Pharmacy, Andalas University, Padang, West Sumatra, Indonesia.

Yohannes Alen currently as a lecturer functional positions at the Faculty of Pharmacy, Universitas Andalas. Graduated from the Faculty of Mathematics and Sciences, Universitas Andalas in 1984 and completed his PhD from Okayama University in 2000. His research interests are in the isolation and elucidation of structures of natural active compounds, pharmacological bioactivity of natural compounds, and phytopharmaca of plants. Currently working as lecturer at the Department of Pharmacy, Faculty of Pharmacy, Universitas Andalas, Padang, West Sumatera, Indonesia.

Salman Umar is a lecture at the Department of Pharmacy, Faculty of Pharmacy, Universitas Andalas Padang, West Sumatera, Indonesia. Got his undergraduate degrees from Andalas University in 1990 and Magisetr Program at Departmen of Pharmacy, ITB Bandung in 1995 then finished his PhD Programm from Departmen Biomedic, Faculty of Medicine Universitas Andalas in 2015. His research interest is in the area of Biopharmaceutic and Biomedical.

Yufri Aldi is a lecturer functional positions at the Faculty of Pharmacy, Universitas Andalas. Graduated from Faculty of Pharmacy Universitas Andalas in 1989, then Master Program at Faculty of Pharmacy Universitas Andalas in 1994 and Doctoral Program in Departement Biomedical, Faculty of Medicine, Universitas Andalas in 2013.The research and expertise are in Farmaco-Immunology. Currently working as head of the Department Pharmacy Doctoral Program, Faculty of Pharmacy, Universitas Andalas.

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