Immunostimulating Activity of Sungkai Leaf Stigmasterol Isolate against Cluster Differentiated 8+ T (CD8+T) from Covid 19 Disease Exposure

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ABSTRACT

Objective: Covid 19 is a disease that infects cells in the airway lining the alveoli. This disease is caused by a virus named SarsCoV2. One way to handle it is to increase immunity. The body itself consists of immune system organs. One component of the immune system that is very important in protecting the body against the SARS-Cov-2 virus attack is Cluster of Differentiation 8+ T (CD8+T). CD8+T is one of the components of the adaptive immune system or often known as the specific immune system and is cytotoxic, so it is called Cluster of Differentiation8+T. One of the plants used by the community to increase endurance is Sungkai (Peronema canescens Jack.). This study aims to see its activity against Cluster of Differentiation 8+ T (CD8+T). Methods: Sungkai leaf powder was extracted using 70% ethanol solvent and evaporated using a rotary evaporator to obtain ethanol extract and fractionated with n-hexanes and isolated to obtain active isolates of sungkai leaves against the immune system. Isolates obtained from the isolation of the active fraction of n hexanes that have been tested as immunostimulants in previous studies. This study used test animals, namely male white mice of the wistar strain as many as 30 mice consisting of 5 heads for each group. Group I is a control group that is not given treatment and groups II, III, IV, V and VI are given exposure to the Sars CoV2 virus antigen that causes Covid 19 disease, namely using one of the Covid19 vaccines, namely the 0.0013 mL Moderna vaccine which is given Intra Muscular. Groups II, III, IV, V are treatment groups induced with Sars CoV2 antigen and given a test preparation, namely sungkai leaf isolate with 3 dose variations, namely 1, 10 and 100 mg/kg bw given orally, and group VI was given stimuno 50 mg/kgbw as a comparison. Results: From the results of immunostimulant testing of sungkai leaf isolate, it was found that stigmasterol has an immunostimulant effect in increasing Cluster of Differentiation 8+ T (CD8+T) in the body given SARS-CoV2 virus antigen. Significant results were seen in the 100 mg/kg bw dose group which increased CD8+T by 53.93 ng/dl. Based on statistical tests conducted in the form of ANOVA tests, CD8+T also showed significant differences with a significance value of <0.05. Conclusion: Stigmasterol isolate of sungkai leaf has the effect of increasing Cluster of Differentiation 8+ T (CD8+T) levels in the body given exposure to SARS-CoV2 virus antigens.

Key words: SARS-CoV-2, Sungkai, Cluster of Differentiation 8+ T (CD8+T), Stigmasterol, Immunostimulant.

INTRODUCTION

Medicinal plants are plants that have medicinal properties and are used as drugs in the healing and prevention of diseases. Indonesia is one of the countries known as a storehouse of medicinal plants. There are about 30,000 species of flora found in Indonesia’s tropical forests. About 9,600 species have been recognized as medicinal. Since ancient times, medicinal plants processed into medicines have been widely used by the Indonesian people, both in the form of single fresh, mixed, and herbs which are better known as traditional medicines.1

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. Several components that are involved in the non-specific immune system are macrophage cells (phagocytic white blood cells) and 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 immune response. Many of these compounds are found in plants that can stimulate these immune response functions which are called immunomodulators.2

The sungkai plant (Peronema canescens Jack.) produces high quality wood, almost comparable to teak wood. From the results of research on the identification of traditional medicinal plants of the Lembak Eight tribe in Bengkulu, it is known that young leaves of Peronema canescens Jack are the raw material for herbal medicines to reduce heat and in tribal medicine, sungkai leaves are pounded and slapper for bruises, sungkai stem water is drunk as a smallpox medicine. The sungkai plant is traditionally used by the Dayak tribe in East Kalimantan as a medicine, among others, as a cold medicine, fever, ringworms, used as a bath for women after childbirth and as a mouthwash to prevent toothache.3

Secondary metabolites contained in sungkai leaf extract include alkaloid, flavanoid, terpenoid-steroid, and tannin compounds.4 The isolation results of n-hexan extract of sungkai leaves obtained terpenoid compounds, UV spectra data with a maximum wavelength of 207, and IR data of active results of n-hexan extract of sungkai leaves obtained terpenoid compounds, UV spectra data with a maximum wavelength of 207, and IR data of active extracts include alkaloid, flavanoid, terpenoid-steroid, and tannin compounds.4 The isolation results of n-hexan extract of sungkai leaves obtained terpenoid compounds, UV spectra data with a maximum wavelength of 207, and IR data of active isolates containing OH (hydroxyl) -CH- aliphatic functional groups, C=O (carbonyl),
Cluster of Differentiation 8+ T cells (CD8+T) require activation and an instruction signal to direct differentiation to the CD8+T pathway. Cells that can recognize their own MHC will be selected for the maturation process which is recognized as positive selection. MHC class I will issue an instruction signal to direct differentiation to the CD8+T pathway.7

Cluster of Differentiation 8+ T cells (CD8+T) require activation and differentiation to become effector T cells that can lyse antigen-infected target cells and tumor cells. Cluster of Differentiation 8+ T cells (CD8+T) recognize antigens exposed by MHC I molecules, because MHC I molecules can be found on body cells that have a nucleus, Cluster of Differentiation 8+ T cells (CD8+T) easily monitor cells for signs of infection. Cluster of Differentiation 8+ T cells (CD8+T) can also be activated as effector T cells after encountering antigens in professional or non-professional APCs and receiving "second signals" from cytokines such as IL-2 IFN γ, and TNF a released by CD4 helper T cells. In the human immune system, Cluster of Differentiation 8+ T cells (CD8+T) will function as cytotoxic/suppressor T cells that interact with MHC class I molecules along with processed antigen fragments. Cytotoxic T cells will destroy other cells by inserting perforin into the target cell membrane so as to produce a porus through the way granzymes (granzymes) are inserted and cause granule-associated osmotic lysis reactions in target cells (the same reaction as the complement membrane attack complex) or through the activity of caspases (capases) to stimulate apoptosis in target cells. These responses are useful for controlling viral infections and malignancies and these cytotoxic/suppressor T cells also regulate other immune responses by releasing soluble factors that act on B lymphocytes to produce antibodies.7

The dynamics of the Cluster of Differentiation 8+ T (CD8+T) lymphocyte response will affect the course of viral infection. The entry of antigen will cause virus-specific Cluster of Differentiation 8+ T (CD8+T) lymphocytes to be activated and will eventually undergo a clonal expansion program. This initial phase of expansion is accompanied by cellular changes consisting of induction of peripheral tissue homing receptors, downregulation of homeostatic cytokine receptors and upregulation of effector molecules such as granzyme B and perforin which are all components of the antiviral Cluster of Differentiation 8+ T (CD8+T) cell response.7

After the expansion phase, some effector cells will die by apoptosis while some surviving cells will become memory cells. Virus-specific memory Cluster of Differentiation 8+ T (CD8+T) cells will proliferate in response to homostatic cytokines such as IL7 and IL 15 that can occur in the absence of antigen, forming a lasting protective mechanism on antigen reexposure.7

**MATERIALS AND METHODS**

**Plant materials**

The materials of this study Peronema canescens Jack. obtained from the Sungkai Ecopark, Lambung Bukik Village, Pauh District, Padang.

**Tools**

The tools used were filter paper, rotary evaporator, a set of column chromatography equipment, vials, TLC Silica desgel 60 F254 (Merck) vessel (1.05554. 0001), dropper pipettes, syringes, measuring cups, animal scales, spatulas, oral needles, analytical scales, containers (bottles), mortar and pestle, surgical scissors, 365 nm UV lamp, incubator, set of centrifuge apparatus, centrifuge tubes, micropipette 10- 100 µL (Socorex), micropipette 100-1000 µL (Socorex), pH meter (Metrohm), eppendorf centrifuge 5418, 1 mL syringe, microtube (Eppendorf), UV-Vis spectrophotometer (Shimadzu), IR spectrophotometer (Shimadzu) and BIO-RAD spectrophotometer.

**Materials**

The materials used were dried and mashed sungkai (Peronema canescens Jack.) leaves, Ethanol (Lichrosolv R (1.11727.2500), Methanol (EMSURER) (1.06009.2500), n-hexane, ethyl acetate, n-butanol, Stimuno 60 mL (PT. Dexta Medica No. Bach 53A4211) Serum (FBS) (Sigma), Silica gel 60 (Merck, 0.063-0.200 mm), (1.07733.1000), Vanillin (Merck) (8.18718.0100), Chloroform (EMSURE R (1.02445.2500), Silica gel 60 (Merck) (0.2-0.5 mm) (1.07733.1000), Mouse CD8 Cell ELISA kit (BT Lab), Physiological NaCl, Na CMC, Vaccine Moderna.

**Dry powder preparation and sungkai leaf extraction**

Sungkai leaf as much as 10 kg were cut into thin pieces with a thickness of 2-3 mm, then dried for 1 week. The dried sungkai leaves were...
mashed using a grinder and obtained a leaf weight of 5 kg. Ethanol extract of sungkai leaves was obtained by extracting the simpilis that had been made using the maceration method. This maceration method was chosen because it does not have to go through a heating process that can damage thermolabile compounds. For the maceration process, 70% ethanol is used as a solvent because the simpilis used has relatively little water content, so 70% ethanol is needed which is known to contain 30% water which functions to break down cell walls so that ethanol penetration into cells becomes faster and more optimal.

The maceration process is carried out with a simpilis: solvent ratio of 1: 10 in a brown bottle container. Simpilis that has been soaked with solvent is stirred then allowed to stand at room temperature for 18 hours with occasional stirring. After maceration, the filtration process was carried out, namely the process of separating the maceration results (macerate) from the pulp using filter paper. This method was repeated 3 times. The obtained macerate was evaporated with the help of a rotary evaporator. The evaporation process is carried out with a temperature of 450C and a pressure of 150 bar until the thick extract of sungkai leaves is obtained.

**Fractionation of sungkai leaf**

Fractionation using n-hexane, ethyl acetate, and n-butanol. A total of 50 g of thick ethanol extract was dissolved in 600 mL of distilled water and then put into a separating funnel with the faucet closed. A total of 200 mL of n-hexane was added to the extract solution and shaken until homogeneous. The n-hexane fraction was separated from the water fraction by opening the separatory funnel faucet and accommodated in an erlenmeyer. The treatment was repeated 2 times with the same solvent. The n-hexane fraction was evaporated with a rotary vacuum evaporator at 40°C to obtain n-hexane extract. The remaining water fraction of n-hexane was added with ethyl acetate solvent as much as 200 mL. The process is done the same as in the n-hexane fraction, so that the ethyl acetate extract is obtained. After that, continued by adding as much as 200 mL of n-butanol to the residual water fraction of ethyl acetate, resulting in n-butanol extract and residual water fraction. The n-hexane, ethyl acetate, n-butanol, and residual water extracts were tested for immunostimulants using the ELISA method and it was found that the fractionation results using n-hexane had higher immunostimulant activity compared to other fractionation results in increasing Cluster of Differentiation 8+ T (CD8+T) levels.

**Characterization of sungkai leaf powder**

Fresh sungkai leaves obtained from the Sungkai Ecopark area, Lambung Bukik Village, Pauh District, Padang City and have been identified at the Herbarium of the Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University (ANDA) Padang Number 102/KID/ANDA/II/2021 with the results of sample identification, namely the species Peronema canescens Jack from the Lamiaceae family. A total of 10 kg of sungkai leaves were dried and 1 kg of fine powder was obtained. The characteristics of the powder obtained are yield of 16.061, drying shrinkage of 10.8968%, and ash content of 6.7090%. Phytochemical tests showed that sungkai leaf extract contained alkaloids, flavonoids, phenolics, saponins, and triterpenoids. And in the extract chromatography test, the RF value of 0.74 was obtained.

**Preparation of animal experiments**

The animals used were male white mice aged 8-12 weeks weighing between 20-30 grams as many as 30 animals. Before being treated, mice were acclimatized for 7 days with adequate food and drink. This aims to adjust the environment, control health and body weight. Animals used are healthy mice, namely body weight during acclimatization does not change more than 10% and visually shows normal behavior.

**Dosage preparation**

Isolates were suspended in 0.5% Na CMC. Na CMC weighed as much as 500 mg and then put in a mortar that already has 10 mL of hot water in it, grind until homogen and then enough water to 100 mL. Dissolve the isolate with Na.CMC solution with a predetermined concentration.

**Treatment of experimental animals**

Mice (Mus musculus BALB/c strain) healthy with a body weight of 20-25 g, totaling 30 heads and randomly divided into each group consisting of 5 heads. Treatment was carried out once a day in the afternoon, for (group I) is a negative control group where mice do not get treatment. Group II is a positive control where mice are only given moderna vaccine at a dose of 0.0013 mL. Group III consists of mice given Stigmasterol isolate at a dose of 1 mg/kg bw, 10 mg/kg bw, and 100 mg/kg bw. Group IV each mice received a comparator in the form of stimuno at a dose of 6.5 mg / kg bw. Treatment to each test animal was carried out for 14 days and on the 15th day the animal was sacrificed by dislocating the neck, blood was taken from the neck. Blood was taken as much as 1 mL left for 30 minutes and then centrifuged for 10 minutes. Afterwards, the Cluster of Differentiation 8+ T was measured using the ELISA method.

**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. Significant results with meaningfulness were taken at p<0.05.

**Ethical approval**

Ethical approval was obtained from the Ethics Committee of the Faculty of Medicine, Andalas University, with ethics letter contract number: 405/UN.16.2/KEP-FK/2021.

**RESULT**

Increasing the dose given also increases the effect on the immunostimulant effect of the isolate. In the results of Cluster of Differentiation 8+ T (CD8+T) showed the highest concentration at a dose of 100 mg / kg bw of stigmasterol isolate. The results of Cluster of Differentiation 8+ T (CD8+T) at a dose of 1 mg/kg bw on stigmasterol 39.61 ng/dl, at a dose of 10 mg/kg bw stigmasterol 40.89 ng/dl, and a dose of 100 mg/kg bw stigmasterol 53.93 ng/dl. Based on statistical tests conducted in the form of ANOVA tests, CD8 + T also showed significant differences with a significance value <0.05 for each treatment given. The results of the duncan test on Cluster of Differentiation 8 + T (CD8 + T) also show that the duncan test that has been carried out shows no significant difference between the positive control and the negative control of the Cluster of Differentiation 8 + T (CD8 + T) protein concentration, the most significant real difference is between the negative control and the administration of stigmasterol isolate at a dose of 100 mg / kg bw.

**DISCUSSION**

The study used leaf of sungkai that had been identified in the Herbarium of the Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University (ANDA) Padang Number 102/ KID/ANDA/II/2021 with the results of sample identification, namely the species Peronema canescens Jack from the Lamiaceae family. Isolation of immunostimulant compounds from active isolates of n-hexan fractionated sungkai leaves using column chromatography. Isolate sungkai leaves in column chromatography using silica gel 60 stationary phase (0.063-0.2 mm) and ethyl acetate: methanol mobile phase isocratically. The isolation results obtained after eludation of
the structure have a compound name in the form of stigmasterol. This active isolate of sungkai leaves will then be tested to see the activity of the isolate to provide immunostimulant effects. From the assay results showed that the characterization and structure elucidation confirmed the isolate used was stigmasterol.

The results of this study based on the average number of Cluster of Differentiation 8+ T (CD8+T) protein concentrations showed that the highest Cluster of Differentiation 8+ T (CD8+T) protein concentration was found at a dose of 100 mg / kgbw on stigmasterol isolate. The concentration of Cluster of Differentiation 8+ T cells (CD8+T cells) given the active isolate of Sungkai leaves compared to the comparator in the form of Stimuno at a dose of 50 mg/kgbw showed that the increase was better using the active isolate of Sungkai leaves. Based on ANOVA statistical calculations, it produces (p<0.05) which means it shows significant results of meaningful differences. Cluster of Differentiation 8+ T (CD8+T) expresses the CD8+T coreceptor and destroys infected cells between dependent MHC-I specific antigens. CD8+T cells can kill cells directly and through induction of apoptosis. Cluster of Differentiation 8+ T (CD8+T) contains abundant azurophilic granules and is able to destroy a variety of tumor cells, infected cells and abnormal cells without prior sensitization. The effective viral response of the host is carried out by the innate and adaptive immune systems through the production of various proinflammatory cytokines, activation of T cells, CD4, and CD8 cells. T cells are important for controlling virus replication, limiting virus spread and clearing infected cells. However, virus-induced tissue damage can lead to overproduction of proinflammatory cytokines, recruitment of macrophages and proinflammatory granulocytes, known as a cytokine storm, which can lead to more severe tissue damage. Plants containing epifriedelanol, lupeol, stigmastanol, triacontane-1-ol, dotriacontane-1-ol, lupeol acetate, deoxyelephantopin, isodeoxyelephantopin, polyphenol luteolin-7, as well as various flavonoids and glucosides can act as immunostimulants because stigmasterol is a compound that has been shown to have health benefits that include lipid-lowering, anti-cancer, anti-inflammatory and anti-allergic effects. Previous studies reported that stigmasterol acts as an anti-inflammatory and anti-arthritis and stigmasterol has the potential to modulate the immune response. Stigmasterol is an unsaturated phytosterol belonging to the class of tetracyclic triterpenes. It is one of the most common plant sterols, found in various natural sources, including vegetable fats or oils from many plants. Currently, stigmasterol has been examined through in vitro and in vivo assays and molecular docking for its various biological activities on different metabolic disorders. Findings show strong pharmacological effects such as immunomodulators in enhancing the specific immune system. Stigmasterol compounds can act as immunostimulants that can improve the work of the immune system, capable of stimulating both specific and non-specific immune functions, especially through NK cells, macrophages, and cytokine induction. Therefore, plants containing this compound can be developed as an alternative therapy in improving the body’s immune system. This immune system can detect pathogenic organisms, from viruses to parasites and worms and distinguish them from normal cells and tissues. As a complex organ composed of specific cells, the immune system is also a separate circulatory system of blood vessels that all work together to eliminate infections in the body. The organs of the immune system are located throughout the body, and are called lymphoid organs. Lymph vessels and lymph nodes are part of a specialized circulatory system that carries lymph fluid, a transparent fluid containing white blood cells, mainly lymphocytes. Lymph nodes contain a network of lymph vessels and provide a medium for immune system cells to defend the body against pathogenic agents. CONCLUSION Sungkai leaf stigmasterol isolate has the effect of increasing Cluster of Differentiation 8+ T (CD8+T) levels in the body given exposure to SARS-CoV 2 virus antigens that cause Covid 19 disease. ACKNOWLEDGMENT The author would like to thank KEMENDIKBUDRISTEK in the Postgraduate Research Program-Doctoral Dissertation Research with contract number 012/ES/PG.02.00.PL/2023 on April 12, 2023. CONFLICTS OF INTEREST There are no conflicts of interest in this study. REFERENCES 1. McKechnie JL, Blish CA. The Innate Immune System: Fighting on the Front Lines or Fanning the Flames of COVID-19? Cell Host Microbe. 2020;27(6):863-9. 2. 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**GRAPHICAL ABSTRACT**

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