Trop J Nat Prod Res, December 2023; 7(12):5420-5423

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Stigmasterol isolated from Sungkai (*Peronema canescens* Jack) leaves Induced Natural Killer Cells in SARS-CoV-2 Antigen Challenged Albino Mice

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ARTICLE INFO

Article history:
Received 11 July 2023
Revised 16 October 2023
Accepted 15 December 2023
Published online 01 January 2024

ABSTRACT

Sungkai leaves have been used to boost the body's immune system. This study evaluated the effect of different doses (1, 10 and 100 mg/kg BW) of stigmasterol isolated from *Peronema canescens* (Sungkai leaves) on Natural Killer cells in male albino mice exposed to Sars-Cov-2 virus antigen. Thirty adult male albino mice used in this experiment were divided into six groups of 5 mice, labelled I-VI. Group I was the uninfected control group, group II (infected and untreated). Groups III-V were exposed to 1.3 x 10^3 mL of SARS-CoV-2 virus Antigen and then treated with 1, 10 and 100 mg/kg bw of stigmasterol. Group VI animals received 6.5 mg/kg bw of Stimuno immunomodulator. After the treatment, the concentration of the Natural Killer (NK) cells in the experimental animals was measured. The different doses of the stigmasterol gave different concentrations of NK cells. At 1 mg/kg BW the mean value was 5.31 ng/mL; at 10 mg/kg BW, it was 6.45 ng/mL; and at 100 mg/kg bw, the value was 6.44 ng/mL at $p<0.05$. The results showed a dose-dependent increase in NK cell concentration in the treatment groups. The highest NK cell concentration (6.46 ng/dL) was obtained in the group treated with 10 mg/kg BW stigmasterol. The study concluded that stigmasterol isolated from the leaves of *Peronema canescens* boosted the NK cell concentration in SARS-CoV-2 antigen-challenged albino mice.

Keywords: *Peronema canescens* Jack., *Natural Killer Cell, Sars Cov 2, Moderna Vaccine, Stigmasterol Isolate*

Introduction

The immune system is the body's defence mechanism that responds to the "attacks" from outside the body. In a chemical or biological assault on the body by antigens, the immune system is usually stimulated to produce cells that directly attack the pathogens or produce antibodies against the invading antigens. This mechanism protects the body from attacks by various microorganisms such as bacteria, viruses, fungi and different disease-causing germs. When the immune system is not optimal, the body becomes susceptible to disease. Several factors affect the immune system, for example, food, environmental stressors, daily lifestyle, age, stress and hormones. Daily intake of a healthy and balanced diet, regular exercise, and a healthy lifestyle are ways to keep the body's immune system at optimum.[1]

Natural killer (NK) cells are important in the body's natural defense against the growth of cancer cells and various infectious diseases, especially viral infections. Most NK cells (95%) can function, and target cells infected with viruses and also initiate the coating of other cells with immunoglobulin G (IgG) to serve as antibody-dependent cell-mediated cytotoxic cells (ADCC).[2]

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Citation: Dillasamola D, Wahyuni FS, Rita RS, Aldi Y. Stigmasterol isolated from Sungkai (*Peronema canescens* Jack) leaves Induced Natural Killer Cells in SARS-CoV-2 Antigen Challenged Albino Mice. Trop J Nat Prod Res. 2023; 7(12):5420-5423. http://www.doi.org/10.26538/tjnpr/v7i12.8

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

NK cells are a subpopulation of lymphocytes larger than T and B lymphocytes and contain specific cytoplasmic granules.[3] NK cells can produce IFN-gamma and TNF-alpha, potent immunoregulatory cytokines. Natural killer (NK) cells in the immune system have a crucial role because they are believed to be the bridge between innate immunity and adaptive immunity.[2]

NK cells are a subset of lymphocytes with one of the surface markers CD16 (a receptor for Fc) and CD56. The surface features of CD16+ and CD56+ without CD3 (CD16+/CD56+CD3-) have been used to ensure that these cells are NK cells that can differentiate between T and B cells. NK cells can kill target cells directly without prior sensitisation and without depending on the major histocompatibility complex (MHC).[4] Apart from being independent of MHC, these cells do not interact with target cells via T cells.[5]

Peronema canescens leaves extract is used in traditional medical practice to boost the immune system. This medicinal plant has efficacy as an immunomodulatory agent or enhancer of the immune system.[6] *Peronema canescens*, at an oral dose of 0.5625 mg/kg BW, has been shown to increase the number of leukocytes by 36%.[7] A study by Sitepu (2013) also showed that ethanol extract from the leaves of *Peronema canescens* exhibited *in vivo* antiplasmodial activity with an ED50 value of 102.88 mg/kg bw.[13] Research findings have shown the presence of different secondary metabolites (flavonoids, phenolic, phytosterols and terpenoids) in Sungkai leaf extracts. Stigmasterol is an unsaturated phytosterol belonging to the class of tetracyclic triterpenes, present in copious amounts in Sungkai leaf. It is one of the most common plant sterols found in various natural sources, including vegetable fats or oils from many plants. Stigmasterol has been examined through *in vitro* and *in vivo* tests and molecular docking for its various biological activities in different metabolic disorders.[8,9,10] The findings of these studies indicate its strong pharmacological effect as an immunomodulator in enhancing the specific immune system.[11]

Plant extracts are used in traditional medicine as decoctions, concoctions, pastes, and powders. The amounts of these

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

phytoconstituents in an extract depend on the extraction method and solvents. This present study aimed to investigate the immunomodulatory effects of *Peronema canescens* leaves on NK cell activity in white mice exposed to Sars Cov-2 virus antigen.

Materials and Method

Collection and Identification of Sungkai Leaves
Samples of Sungkai (*Peronema canescens* Jack) were obtained in February 2021 from *UPTD Rumah Potong Hewan Aia Pacah* Padang City, West Sumatera. The plant sample was identified and validated by Dr Nurainas of Andalas University Herbarium (ANDA), Department of Biology, Faculty of Mathematics and Natural Science, Universitas Andalas, Padang City, West Sumatera. A voucher specimen number 102/K-ID/ANDA/II/2021 was assigned.

Ethicals approval
Approval for using the experimental animals in this study was obtained from the Ethics Committee of the Faculty of Medicine, Andalas University, with ethical approval number No: 405/UN.16.2/KEP-FK/2021. Thirty adult male albino mice used in this study were housed in clean plastic cages, allowed to rodent pellets and water *ad libitum*. The OECD guidelines for the care and use of experimental animals were strictly followed in this study.

Extraction of plant material
Sungkai leaf (3 kg) was cut into thin pieces and dried under shade for a week. The dried sungkai leaves were ground to obtain 1 kg of the powdered sample. The powdered sample (1 kg) was extracted by maceration with 70% ethanol (5 L) for 3 days. The extract was filtered with Whatman No. 1 filter paper, and the filtrate was concentrated to dryness using a rotary evaporator (Buchi R-210 Rotavapor) at 40℃. The crude extract obtained was stored at 4℃ in a refrigerator until further use.

Fractionation
The ethanol crude extract (50 g) was dissolved in 600 mL of distilled water and successively fractionated with n-hexane (200 mL x 2) and ethyl acetate (200 mL x 2). The individual fractions were collected and concentrated with a rotary evaporator at 40℃ and stored until further use.
The crude n-hexane fraction was loaded onto a chromatographic column (60 x 5 cm) packed with normal silica gel 60 (63 - 200µm) and eluted isocratically with a solvent mixture of ethyl acetate: methanol to obtain 10 fractions (F1-F10). Eluates were monitored with a TLC plate (F_{245}, Merck, US) and a 254 nm UV lamp (Camag, Switzerland). Stigmasterol was separated from F4-F6 after repeated cc as a white fluffy powder (24 mg). The compound was identified spectrophotometrically by comparison with the literature data.[14]

Dose and Induction
The test sample used was active isolates of Sungkai leaves (stigmasterol) at doses 1, 10, and 100 mg/kg body weight. The inductor used is the Sars Cov 2 (moderna) vaccine 1.3 x 10^3 mL.
The treatment for animal test
Test animals were grouped into six groups consisting of 5 mice each. Group I was the uninfected control group, group II (infected with 1.3 x 10^3 mL of SARS-CoV-2 virus Antigen and untreated). Groups III-V were exposed to 1.3 x 10^3 mL of SARS-CoV-2 virus Antigen and then treated with 1, 10 and 100 mg/kg bw of stigmasterol. Group VI animals received 6.5 mg/kg bw of Stimuno immunomodulator. The treatment continued from day 1-14.

The Collection of Blood Serum for Measurement of NK Cells
Blood was taken from the animals' neck arteries (using the guillotine technique) on day 15 of the experiment into test tubes containing EDTA and centrifuged for 30 minutes at 3000 rpm. Serum was obtained, and the NK cell concentration was tested using the ELISA method.[7]

Data analysis
The data obtained from the research results were analysed statistically using the one-way Analysis of Variance (ANOVA) method and then Duncan's analysis using IBM SPSS statistical software version 24.

Result and Discussion

The results obtained from the test animals show that the concentrations of natural killer (NK) cells increased with increased concentration of Sunkai leaf sample (stigmasterol). The average NK cell concentration at 1 mg/kg bw of stigmasterol was 5.31 ng/mL, 10 mg/kg bw 6.45 ng/mL, and 100 mg/kg bw 6.44 ng/mL (Table 1). These values are considered significant from ANOVA analysis which shows $p<0.05$, indicating a significant difference in each treatment (Table 2). The Duncan test also showed that the positive and negative controls significantly differed in the Natural Killer cells (NK cells) concentration. The negative control with stigmasterol at 10 mg/kg bw showed the most significant difference (Table 3).
The process of making sungkai leaves extract (*Peronema canescens* Jack.) begins with collecting fresh sungkai leaves, cleaning them from impurities, washing them with running water and then air-drying them until they become dry simplicia, then after the dried simplicia is ground using a grinder and powdered simplicia is obtained. Sample maceration was carried out using 70% ethanol.[8] From 2.4 kg of simplicia powder, the extract yield was 387.5 g at 16.14%. Sungkai leaves powder (Peronema canesens Jack) was extracted using a multilevel maceration method to obtain non-polar, semi-polar, and polar fractions. The plant material was successively macerated, starting with non-polar n-hexane at a ratio of 1: 10, followed by ethyl acetate (1: 10) and then butanol (1: 10). The mother liquor (aqueous extract) was also obtained. Each filtrate was concentrated to dryness using a rotary evaporator to obtain the different crude extracts. The sungkai leaves extract (*Peronema canescens* Jack.) was standardised. The drying shrinkage of the extract obtained was 8.91%, which complied with the provisions of the Indonesian Herbal Pharmacopoeia, where the drying shrinkage of gotu kola herb extract was not more than 10%. The total ash content of leaves extract sungkai (*Peronema canescens* Jack.) was 3.66%, which also fulfils the provisions of the Indonesian Herbal Pharmacopoeia, which stipulates that the total ash content of the condensed extract of sungkai leaves should not be more than 10%.[8]
The organoleptic examination aims for a simple initial recognition of the sungkai leaf extract used. The extract was viscous, with a distinctive odour, blackish-brown colour and bitter taste. The phytochemical test showed the presence of flavonoids, phenolics, saponins and terpenoids.[15]
Analysis of active isolates from Sungkai leaves was carried out using hexane and ethyl acetate eluents at a ratio (9:1). The plate was observed at the wavelengths of 254 nm and 366 nm, followed by spraying with 10% H_2SO_4. The (retention factor) Rf value obtained for stigmasterol was 0.24.
The concentration of NK cells was carried out by collecting serum from the blood of mice that had been given active isolate from Sungkai leaves at doses of 1, 10 and 100 mg/kg bw and exposed to Sars Cov 2 virus antigen, the blood was centrifuged at 3000 rpm then the serum was taken for further analysis using ELISA. The highest concentration of NK cells obtained was 6.45 ng/µL in the 10 mg/kg bw group, and the lowest concentration was found in the negative control group, namely 3.36 ng/µL (figure 1). Data analysis was done using one-way ANOVA, where the p-value <0.05 was considered significant. The result shows a concentration-dependent increase in the NK cells in the treatment groups. Natural killer (NK) cells play a role in the innate immune system's mechanism, recognising and killing virus-infected and tumour cells.[9] NK cells are known to be the front line capable of rapidly mediating the response of infected cells. Recent findings indicate that NK cells can also regulate adaptive immune responses. The role of NK cells as effectors against virus-infected cells is well understood.[10,16]
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

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

many plants. Stigmasterol has been examined through *in vitro* and *in vivo* tests and molecular docking for its various biological activities in different metabolic disorders. The findings indicate a strong pharmacological effect as an immunomodulator in enhancing the specific immune system.[11]

Stigmasterol can act as an immunostimulant to enhance the immune system's work, stimulating specific and non-specific immune functions, particularly through NK cells, macrophages, and cytokine induction. Therefore, plants containing this compound can be developed as alternative therapies to boost the body's immune system. The immune system can detect pathogenic organisms, from viruses to parasites to worms, and distinguish them from normal cells and tissues. As a complex organ consisting of specific cells, the immune system is also a separate circulatory system of blood vessels that work together to eliminate infection 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 special circulatory system that carries lymph, a transparent liquid containing white blood cells, especially lymphocytes. Lymph nodes contain a network of lymph vessels and provide a medium for immune system cells to defend the body against pathogenic agents.[12]

Table 1: Protein concentrations of Natural Killer cells (ng/µL) from negative control, positive control, stigmasterol, and comparison tests

Group	I	II	III	IV	V	Average	SD
Control -	3.12	3.48	3.54	3.29	3.41	3.36	0.14
Control +	4.33	4.42	4.59	4.94	3.74	4.40	0.39
Dose I	5.03	5.73	5.03	5.38	5.38	5.31	0.26
Dose II	5.79	6.76	6.69	6.28	6.73	6.45	0.37
Dose III	6.51	6.98	5.64	6.75	6.31	6.44	0.46
Stimuno	6.93	7.8	6.49	7.37	6.73	7.06	0.52

Tabel 2: ANOVA test of Natural Killer (NK) Cell concentration from negative control test, positive control, stigmasterol, and comparison.

ANOVA					
NK Cell					
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	55.971	8	6.996	33.737	.000
Within Groups	7.466	36	0.207		
Total	63.437	30			

Table 3: Duncan tests Natural Killer Cell concentration (NK cells) from negative control, positive control, stigmasterol, and comparison

NK Cell							
Duncan							
Treatments	**N**	**Subset for alpha = 0.05**					
		1	**2**	**3**	**4**	**5**	**6**
K-	5	3.3680					
K+	5		4.4040				
BI	5			5.3100			
BIII	5					6.4380	
BII	5					6.4500	
Stimuno	5						7.0640
Sig.		1.000	0.596	0.355	0.114	0.194	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Conclusion

There was an increase in NK cell concentration in the treatment groups. The highest NK cell concentration (6.46 ng/dL) was obtained in the group treated with 10 mg/kg BW stigmasterol. This study concluded that stigmasterol isolated from *Peronema canescens* leaves increased NK cell concentration in albino mice challenged with SARS-CoV-2 antigen.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgment

The author would like to thank KEMENDIKBUDRISTEK in the Postgraduate Research-Doctoral Dissertation Research Program with contract number 012/E5/PG.02.00.PL/2023.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

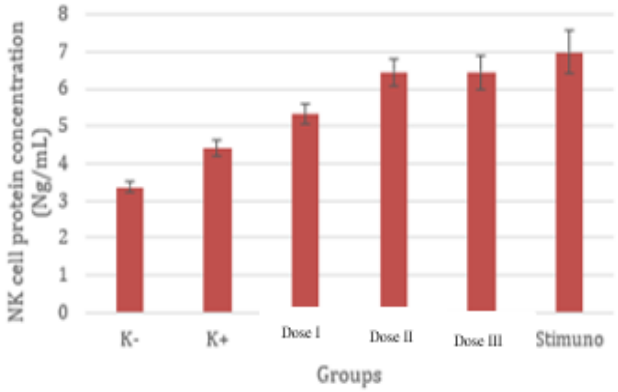

Figure 1: Natural Killer Cell concentrations (NK cells) from negative control, positive control, stigmasterol, and comparison tests.

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