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# Preliminary study of immunomodulatory effects of fermented garlic honey on non-specific immune responses



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# **ABSTRACT**

**Introduction:** Honey and Garlic is recognized to offer several health advantages due to its numerous constituents, one of which being flavonoids. Flavonoids' pharmacological activity includes antibacterial, antioxidant, antifungal, antiviral, and immunomodulatory properties. Honey's immunomodulatory action plays a vital role in immune system boosting. The purpose of this study is to determine the immunomodulatory effect of fermented garlic honey, which is considered to have a synergistic effect, using two alternative techniques.

Methods: Macrophage phagocytic activity is the first approach, while the delayed type hypersensitivity is the second. Each method used 30 male rats (*Rattus norvegicus*) divided into 5 groups, where each group consisted of 6 rats. Group I negative (Placebo 0,005 g/kg BW), group II positive (*Stimuno* 0,005 g/kg BW), group III FGH dose I (1,5 g/kg BW), group IV FGH dose II (3 g/kg BW), group V FGH dose III (4,5 g/kg BW). The dosage form was orally given for 21 days. The Macrophage phagocytic activity method measured %activity of macrophage phagocytosis using a microscope. The rats were injected with an antigen which is *Staphylococcus aureus* by intraperitoneal route.

Results: The Delayed Type Hypersensitivity was measured volume change of the rat's feet at the 4, 24, and 48 hours using a plethysmometer. Based on the results, fermented garlic honey had an immunomodulatory effect. The dose 2 (3 g/kg BW) has the highest effectiveness as an immunomodulator. Fermented Garlic Honey may be concluded to be efficient as an immunomodulator by enhancing the macrophage phagocytic activity indicator and lowering the edema volume of the rat.

Conclusion: Fermented garlic honey can increase the immune system and can be used as an immunostimulant. The most effective dose of fermented garlic honey as immunomodulator is dose 2 was 3 g/kg BW.

**Keywords:** fermented garlic honey, immunomodulator, Macrophage Phagocytic Activity, Delayed Type Hypersensitivity. **Cite This Article:** Wulansari, D.D., Kirtishanti, A., Wulandari, D.D., Santoso, A.R.P., Christy, E.A., Hiskiati, L., Winarni, D. 2023. Preliminary study of immunomodulatory effects of fermented garlic honey on non-specific immune responses. *Bali Medical Journal* 12(3): 3370-3375. DOI: 10.15562/bmj.v12i3.4428

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#### **INTRODUCTION**

Immunity is a protective mechanism against disease, particularly infectious diseases. The immune system is a collection of cells, tissues, and chemicals that have a role in infection defense. In the meanwhile, the immune response is a coordinated reaction of cells and chemicals in response to infection. The host's antigen-protective systems are classified as innate immunity, which gives rapid antigen protection, and adaptive immunity, which develops more slowly than innate immunity but provides more specific protection against infection. The body's natural immune system, which consists of cells and systems, works to defend host cells from illnesses brought on by microbes.1

The main elements of non-specific

immunity are phagocytic cells like polymorphonuclear cells, macrophages, and natural killer (NK) cells, as well as physical and chemical defenses like epithelium and antimicrobial substances produced on the epithelial surface, different types of blood proteins like complementary system components, inflammatory mediators, and various types of cytokines. The phagocytosis process, which is carried out by macrophages, is one method of destroying microorganisms. Bone marrow promonocytes give rise to macrophages, which later develop into blood monocytes and persist in the tissues as mature macrophages with a mononuclear phagocyte system. One type of non-specific immune cell is the macrophage. Circulating monocytes

and tissue macrophages make up mononuclear phagocytes. In all bodily tissues, phagocytic cells may survive for a very long time.2 Macrophages are dispersed throughout connective tissue and close to the basement membrane of tiny blood arteries, with concentrations that are particularly high in the liver's Kupffer cells and the lungs' alveolar macrophages. Because macrophages have dispersed throughout the body, they are employed in the phagocytic activity test, which counts the number of macrophages that phagocytize antigens such as Staphylococcus aureus germs. Furthermore, the macrophage phagocytic activity test is one of the immunological parameters, thus macrophages, which are part of the immune system, are

employed in assessing the body's immune capabilities. Macrophages are bone marrow promonocyte-derived cells that differentiate into blood monocytes before remaining in the tissues as mature macrophages with a variety of functions.<sup>3</sup>

Fermented garlic honey fermentation process that involves raw honey and garlic, this fermentation process involves the natural yeast in honey. It has enormous benefits, the safety tolerance is good in the community. Fermented garlic has a higher antioxidant power. Honey and garlic contain immunomodulatory properties that help to boost the immune system. Honey has the ability to treat COVID-19 as an anti-inflammatory by reducing the action of mast cells, including histamine and cytokines in inflammation and pain, according to prior study.4 Meanwhile, garlic (Allium sativum) has the potential to be used as an antiviral treatment for COVID-19 by inhibiting Mpro SARS-CoV-2 through H-binding with proteases.5 However, honey and garlic continue to be less effective immunomodulators, as seen by the rising frequency of COVID-19 cases.6

So, it was done through study by mixing honey and garlic in the intention of having a synergistic effect to strengthen the immune system. The fermentation technique is used to combine honey and garlic because fermented items may boost the immune system by making the intestines stronger or healthier. Fermented items have the benefit of generating products with a particular taste, fragrance, and texture.7 The fermentation process of mixing honey and garlic lasts 5 weeks, during which time garlic is extracted. Because research data on fermented garlic honey is still few, this study was carried out to provide an update and to demonstrate the usefulness of fermented garlic honey as an immunomodulator.8 In light of the reasoning provided above, the researchers made the decision to investigate the immunomodulatory properties of fermented garlic honey and how it reacts to non-specific immunity. The purpose of this study is to determine the immunomodulatory effect of fermented garlic honey, which is considered to have a synergistic effect, using two alternative techniques.

#### **METHODS**

#### **Study Design**

This type of research is experimental using animals in vivo experiment. Experimental research is a research with intervention or treat a variable. The design of this research is Post Test Only with Control Group Design. This study used five groups, namely the positive control group, negative, and three treatment groups. The raw honey used is from Wild Forest Honey produced from Apis dorsata bees on monoflora nectar and Acacia mangium extrafloral nectar in PT. WKS Bayung Lincir, South Sumatra. Garlic obtained from one of the garlic distributors in Surabaya. Male white rats (Rattus norvegicus) obtained from the Toxicology Laboratory of the Faculty of Pharmacy, University of Surabaya. Stimuno obtained from one of the pharmacies in Surabaya.

# Macrophage Phagocytic Activity Test Material

Fermented garlic honey, *Staphylococcus aureus* bacteria, nutrient agar, nutrient broth, NaCl physiological 0.9%, chloroform, methanol, giems, and immersion oil.

# Delayed Type Hypersensitivity Test Material (DTH)

The antigen used in this study was sheep red blood cells (SDMD) obtained from the Faculty of Veterinary Medicine, Universitas Airlangga, PBS (Phosphate Buffered Saline) pH 7.2.

#### **Data Collection Procedures**

#### Fermented Garlic Honev

Prepare 12 cloves of garlic that have been peeled, washed, and crushed and then prepare 1 1/2 cups of raw honey. Put the crushed garlic into the jar, then pour the honey over the garlic and stir until combined, make sure all the garlic is coated or submerged in the honey. Cover the jar and save it at room temperature for 3 days. Open and remove the lid of the jar to release the gas from the jar. Stir in the garlic and honey. There will be small bubbles that indicate the fermentation process has started. Close the jar again, stirring every two days, at least 1 week before consumption. Honey and garlic fermented for 5 weeks. Fermented honey garlic can be used for 1 month at room temperature.

## **Rats Adaptation**

Rats were adapted to the environment for 14 days in order for the mice to adapt to the new environment. This adaptation process is carried out so that the mice do not feel strange, stressed, or depressed so that it will affect the test results and data interpretation. After the adaptation process, the mice were randomized into 5 groups.

# **Macrophage Phagocytic Activity Test** Treatment

There were 5 groups, namely group 1 was given 0.005 g/kg BW placebo, group 2 was given 0.005 g/kg BW stimulation, group 3 was given FGH at a dose of 1.5 g/kg BW, group 4 was given FGH at a dose of 3 g/kg BW, and group 5 was given FGH at a dose of 4.5 g/kg BW. The process of giving placebo, *stimuno*, and FGH for 21 days.

#### Preparation of Bacteria Suspension

The SA bacteria were regenerated on 50 ml NA media, then incubated for 24 hours at 37°C. After the incubation process, the growing bacterial colonies were transferred into NB media using the aseptic method using a sterile loop. Then bacteria was taken from pure nutrient agar (NA) culture, grown in 50 ml of nutrient broth media and incubated at 37°C for 24 hours. Then it was diluted using new NB media while measurements were made using the McFarland method to obtain a bacterial density of 109. Diluted with 0.9% physiological NaCl solution was carried out to obtain a bacterial count of 109.

#### Observation

The next day after the process of administering the test material, each rat was infected with 0.5 mL of SA bacterial suspension by intraperitoneal injection, then left for one hour. The rats were euthanized with ether and then the abdomen was dissected using sterile scissors and tweezers. Peritoneal fluid is taken using a pipette, then the %activity parameter is calculated. Peritoneal fluid was smeared on an object glass and fixed with methanol for 5 minutes, then stained with Giemsa, allowed to stand for

20 minutes, and then rinsed with water. Observed under a microscope with a magnification (10x-40x) using immersion oil. Calculated the activity of macrophage

phagocytosis. The phagocytic activity of macrophages is determined based on the number of active phagocytic cells that

carry out phagocytosis in 100 phagocytic

The formula to count %activity:

%activity =  $\frac{\text{(number of phagocytoced macrophages)}}{\text{(the number of macrophages counted (100)}} \times 100\%$ 

# Delayed Type Hypersensitivity Test Material (DTH)

Trial Stage

Group I is a negative placebo group (0.005 g/kg BW), group II is a positive stimuno dose (0.005 g/kg BW), group III is a group I (fermented garlic honey) dose (1.5 g/kg BW), group IV is a group II (fermented garlic honey) dose (3 g/kg BW), group V was group III (fermented garlic honey) dose (4.5 g/kg BW). The treatment process was 21 days. On day 16, all the groups were induced with SBRC (sheep red blood cells) 10% v/v antigen as much as 1 mL intraperitoneal. On day 21, all the groups were induced again with SBRC antigen 10% v/v as much as 0.1 mL intraplantar. The measurement of the leg volume of the test animals was carried out on day 21, it measured the rat's leg before induced. After that the rats were induced by 10% v/v SBRC antigen intraplantar at 4, 24 and 48 hours using a plethysmometer. After induced, the rat's leg measured again. By means of each left hind leg of the test animal being marked in a circle with a marker, then measuring the volume of the left leg of the test animal by immersing it in a plethysmometer to the extent of a circle mark, seeing and recording the volume that is read on the device.

#### Antigen Preparation

The antigen used was a 10% Sheep Red Blood Cell (SRBC) suspension. SRBC was obtained from the Faculty of Veterinary Medicine, Universitas Airlangga. The process of making 10% SDMD suspension is carried out in the Biopharmaceutical Laboratory. Based on previous research, sheep red blood cells (SRBC) were accommodated in clean and dry containers containing EDTA as an anticoagulant. Separate the sheep's red blood cells and their plasma by centrifugation at 3000 rpm for 15 minutes. The upper layer in the form of plasma was discarded and the lower layer in the form of red blood cells was added

with PBS (Phosphate Buffered Saline) pH 7.2 as much as three times the volume of the remaining SDMD and inverted several times and then centrifuged again. Washing is done at least 3 times until the top layer is completely clear and colorless. After completion, the PBS was separated and 100% SDMD was obtained, then the same amount of PBS was added to obtain a 50% SDMD suspension. To obtain a 10% SDMD suspension, 2 mL of 50% SDMD suspension was pipetted, and 8 mL of PBS was added.

#### **Data Analysis**

Macrophage Phagocytic Activity

The research analyzed was using the SPSS (Statistic Product and Service Solution) version 26 program. The data was homogeneous and normally distributed, then it was analyzed by one-way ANOVA test and followed by the LSD test (P < 0,05).

## Delayed Type Hypersensitivity (DTH)

The research analyzed was using the SPSS (Statistic Product and Service Solution) version 26 program. The data was not homogeneous, then it was analyzed by Kruskal Wallis and followed by the Mann-Whitney test. The analysis was carried out with a significant value of 0.05.

## **RESULTS**

# **Macrophage Phagocytic Activity**

Based on data analysis using the one-way ANOVA test followed by the LSD test in the SPSS program, the macrophage phagocytic activity data showed normal distribution based on the P>0.05 requirement.

## **Delayed Type Hypersensitivity (DTH)**

Based on the results of the Kruskal Wallis test and continued with the Mann Whitney test, the activity data for the difference in the swelling of the left leg swelling of the rats as a response to DTH shows that the data are as follows:

#### **DISCUSSION**

The fermentation process of fermented garlic honey occurs for 5 weeks, characterized by not foaming, the consistency of the honey becomes thinner and begins to blacken, and the garlic goes down to the bottom of the jar. The reaction of the FGH fermentation process is described below.

Sugar + yeast  $\rightarrow CO_2 + C_2H_5OH + energy$ 

The fermentation process occurs through the liquid garlic combined with enzymes and microbes in honey to be fermented. Yeast cells (from the genus *Zygosaccharomyces*) from honey will degrade sugars in honey and garlic, especially glucose and fructose into alcohol, namely ethanol (C2H5OH), acetic acid and carbon dioxide.<sup>9,10</sup>

In the macrophage phagocytic activity test, antigens were used in the form of Staphylococcus aureus which is gram positive and extracellular bacteria. In dealing with this antigen, there are two suspected mechanisms. The first suspected mechanism is fermented garlic honey as an immunostimulant. FGH stimulates the production of B lymphocytes to produce antibodies. Antibodies will stimulate monocytes, so that monocytes will secrete proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-10. These proinflammatory cytokines increase phagocytic activity, besides that inflammation makes capillary blood permeability increase. 11,12 Phagocytic cells such as macrophages will come out into the peritoneal cavity and phagocytize Staphylococcus aureus. SA will have internalization macrophages, into phagosomes will fuse with lysosomes, then SA is killed by enzymes. The second suspected mechanism is fermented garlic honey as an antibacterial. The presence of phenolic derivatives in honey and allicin in garlic in fermented garlic honey causes inhibition of the production of lipid synthesis of Staphylococcus aureus which

Table 1. Value of Macrophage Phagocytosis Activity Percentage Giving FGH at Various Dose Levels

0/ = ==================================	Treatment					0/ Maan I CD
%activity	P1	P2	Р3	P4	P5	%Mean ± SD
I	50	54	58	58	55	$55 \pm 3.317$ <sup>#</sup>
II	73	78	72	77	78	$75.6 \pm 2.881^*$
III	73	71	77	76	71	$73.6 \pm 2.793^*$
IV	78	86	89	85	85	$84.6 \pm 4.037^{*\#}$
V	77	86	82	87	83	$83 \pm 3.937^{*\#}$

Description:

(\*) = Significantly different when compared to P1 (negative group)

(#) = Significantly different when compared to P2 (positive group)

P1 = Group 1 negative control (0.005g/kg BW placebo)

P2 = Group 2 positive control (0.05g/kg BW stimulus)

P3 = Group 3 (1 FGH dose is 1.5 g/kg BW)

P4 = Group 4 (2nd dose of FGH is 3 g/kg BW)

P5 = Group 5 (3 doses of FGH is 4.5 g/kg BW)

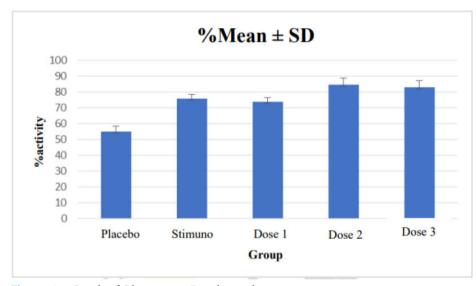


Figure 1. Graph of Observation Results % phagocytic activity.

results in damage to the structure of cell membranes. This damage causes phenol to enter the cell wall and cell membrane of bacteria. Furthermore, there is denaturation of the proteins that make up the protoplasm of bacteria, then bacterial metabolism is inactive. This causes inhibition of growth and development of *Staphylococcus aureus* bacteria. <sup>13,14</sup>

The mechanism of edema in a rat starts from the skin of a normal rat induced by red blood cell antigen (SRBC) containing antigenic lipopolysaccharide. Causes damage to cell membrane enzymes resulting in tissue phospholipase A2 activation, thus the cell membrane phospholipid layer will undergo conversion to arachidonic acid then arachidonic acid will undergo metabolism through the Cyclo-oxygenase (COX 1,2) pathway and

release inflammatory mediators such as PGE2, PGI2, PGD2, PGF2, TXA2. It makes widening of the pores which causes increased membrane permeability and activated macrophages release cytokines. Then cytokines, plasma proteins and plasma fluid will come out through the open pores towards the extracellular and there will be an accumulation of extracellular tissue full of exudate which causes the tissue to be full and edema occurs.15 There is a suspected mechanism of fermented garlic honey as an antiinflammatory to make the edema go down. Normal skin is induced by red blood cell antigen (SDMD) containing antigenic lipopolysaccharide, which causes damage to cell membranes. Phospholipase A2 enzyme gets activated, the phospholipid layer of the cell membrane will undergo

conversion to arachidonic acid. Fermented garlic honey will inhibit arachidonic acid metabolism so that arachidonic acid cannot bind to Cyclo-oxygenase (COX 1,2) and cannot release inflammatory mediators such as PGE2, PGI2, PGD2, PGF2, TXA2 which results in normal pores and membrane permeability were decrease. Fermented garlic honey also inhibits macrophages from binding to antigenic lipopolysaccharides so that they cannot release cytokines. Then cytokines, plasma proteins and plasma fluids will be reabsorbed by the body and reduce edema. <sup>16</sup>

The comparative immunomodulator (positive control) was stimuno which contained Phyllanthus niruri L herb extract. Phyllanthus niruri L can stimulate the human immune system, the content of flavonoid compounds. In macrophage phagocytosis activity method, mechanism of Phyllanthus niruri L extract as an immunomodulator is to activate NK cells to stimulate the production of interferon-y. IFN-y is the main cytokine of MAC (Macrophage Activating Cytokine) and has a role in cellular immunity.<sup>17,18</sup> In the Delayed Type Hypersensitivity method, rats normal skin is induced by red blood cell antigen (SRBC) containing antigenic lipopolysaccharide. Resulting in damage to cell membranes resulting in activation of the tissue phospholipase A2 enzyme so that the phospholipid layer of the cell membrane will undergo conversion to arachidonic acid. Stimuno will inhibit the metabolism of arachidonic acid so that arachidonic acid cannot bind to Cyclo-oxygenase (COX 1,2) and cannot release inflammatory mediators such as PGE2, PGI2, PGD2, PGF2, TXA2 which causes the pores to return to normal and membrane permeability decreases. The stimulus also inhibits macrophages from binding to antigenic lipopolysaccharides so that they cannot release cytokines. Then cytokines, plasma proteins and plasma fluids will be reabsorbed by the body and edema will decrease.19

Based on the macrophage phagocytic activity method and Delayed Type Hypersensitivity method, the first dose of FGH was 1.5 g/kg BW, second dose of FGH was 3 g/kg BW and third dose of FGH was 4.5 g/kg BW had similar effectiveness

#### **Delayed Type Hypersensitivity (DTH)**

Table 2. Value of Macrophage Phagocytosis Activity Percentage Giving FGH at Various Dose Levels

Group	Mean volume (ml) rat's leg ± SD time (hour)				
Group -	T0	T4	T24	T48	
I Negative (Placebo)	$0.04 \pm 0.00$	$0.09 \pm 0.01$	0.12±0.01	0.11±0.01	
II Positive (Stimuno)	$0.03 \pm 0.00$ *	0.07 ±0.01*	0.08±0.01*	$0.06\pm0.00^*$	
III FGH dose 1.5 g/kg BW	$0.03 \pm 0.00$ *	0.07±0.01*	0.08±0.01*	0.07±0.00*	
IV FGH dose 3 g/kg BW	$0.03 \pm 0.01^*$	$0.08\pm0.01^{*a}$	0.08±0.01*	0.06±0.01*	
V FGH dos3 4.5 g/kg BW	$0.03 \pm 0.01^*$	0.08±0.01*	0.09±0.00*	0.07±0.00*a	

Note:

T0 = Measurement of the initial leg volume of experimental animals

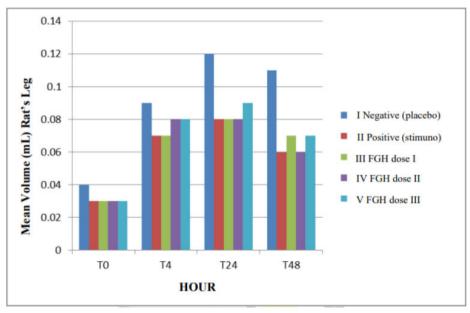
T4 = Measurement at 4 hours after being induced by antigen

T24 = Measurement at 24 hours after antigen induction

T48 = Measurement at 48 hours after antigen induction

(\*) = Data is significantly different (P < 0.05) with negative control group

(a) = The data is significantly different (P<0.05) with the positive group (Stimuno)



**Figure 2.** The graph of the measurement results of the average change in the volume of the rat's leg after administration of the sheep red blood cell antigen (SBRC) 10% v/v.

Note:

Group I negative = Placebo 0.005 g/kg BW Group II positive = Stimuno 0.005 g/kg BW

Group III dose I = Fermented Garlic Honey dose 1.5 g/kg BW Group IV dose II = Fermented Garlic Honey dose 3 g/kg BW Group V dose III = Fermented Garlic Honey dose 4.5 g/kg BW

with positive control which is *stimuno*. At dose 2, which is 3 g/kg BW, it has a higher effectiveness as an immunomodulator which is supported by an increase in the % activity parameter in the macrophage phagocytosis test and the parameter for decreasing the volume of rat foot edema in the DTH test.

#### CONCLUSION

Fermented garlic honey dose 1 was 1.5 g/kg BW, dose 2 was 3 g/kg BW and dose 3 was 4.5 g/kg BW in rats can increase the phagocytic activity of macrophages and can decrease the edema volume from rats. Fermented garlic honey can increase the immune system and can be

used as an immunostimulant. Based on the results of the study, FGH doses of 1.5 g/kg BW, 3 g/kg BW and 4.5 g/kg BW in rats had the same effectiveness as stimuno. However, at a dose of 3 g/kg BW FGH had the highest macrophage phagocytic activity and decreasing leg volume edema in rats. It can be concluded that the most effective dose of fermented garlic honey as immunomodulator is dose 2 was 3 g/kg BW. Further research can be carried out at the molecular level such as ELISA. For macrophage phagocytosis activity method is cytokines (TNF-α, IL-1, dan IL-6) and for Delayed Type Hypersensitivity is antibody titers (TNF, IL-1, IL-6). Further studies are needed to evaluate more deeply various factors that affect the immunomodulatory effects of fermented garlic honey on nonspecific immune responses.

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#### **Conflict of Interest**

No potential conflict of interest relevant to this article was reported.

#### **Author Contribution**

All authors similarly contribute to the think about from the investigate concepts, information acquisitions, information investigation, factual investigations, changing the paper, until detailing the consider comes about through publication.

#### **Ethical Consideration**

Ethical approval was obtained from The Research Ethics Committee of Universitas Surabaya (No.46/EC-KEP-US/I/I2021).

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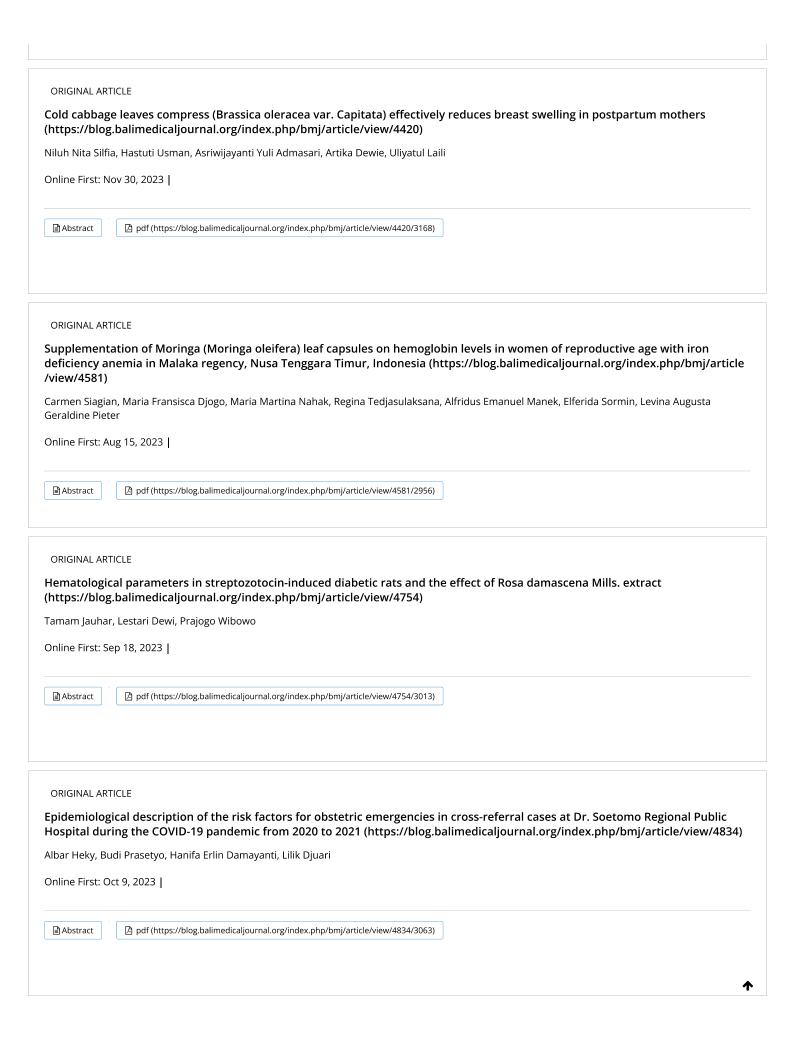
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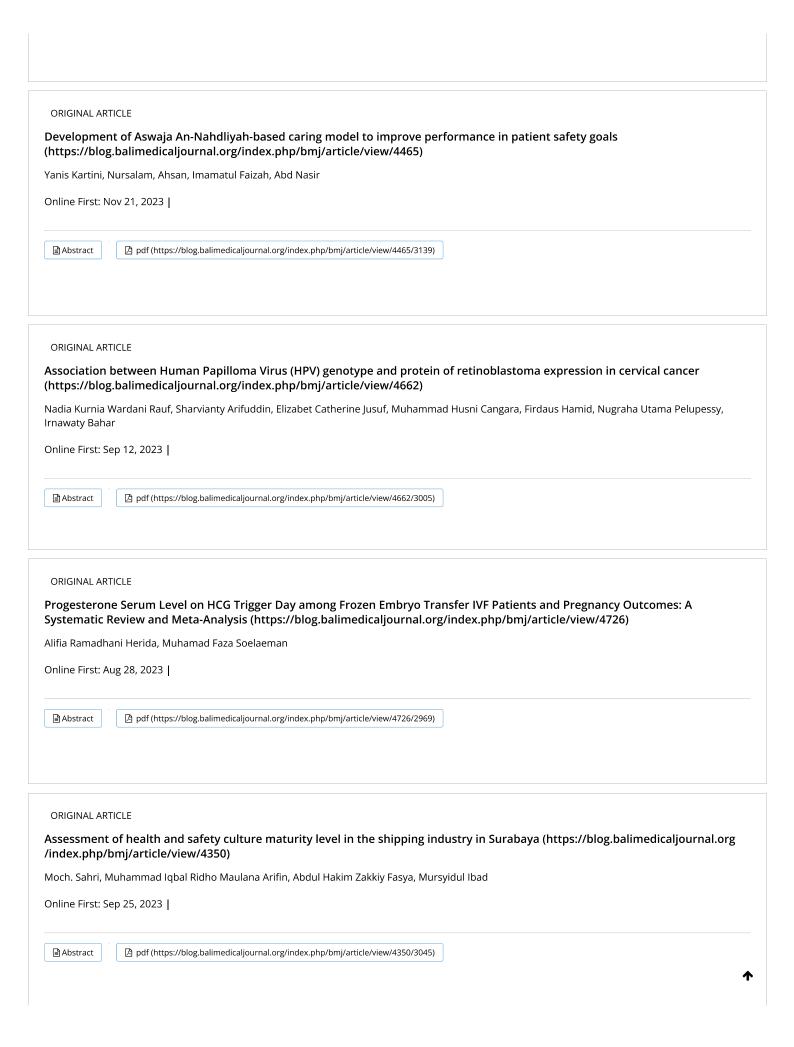
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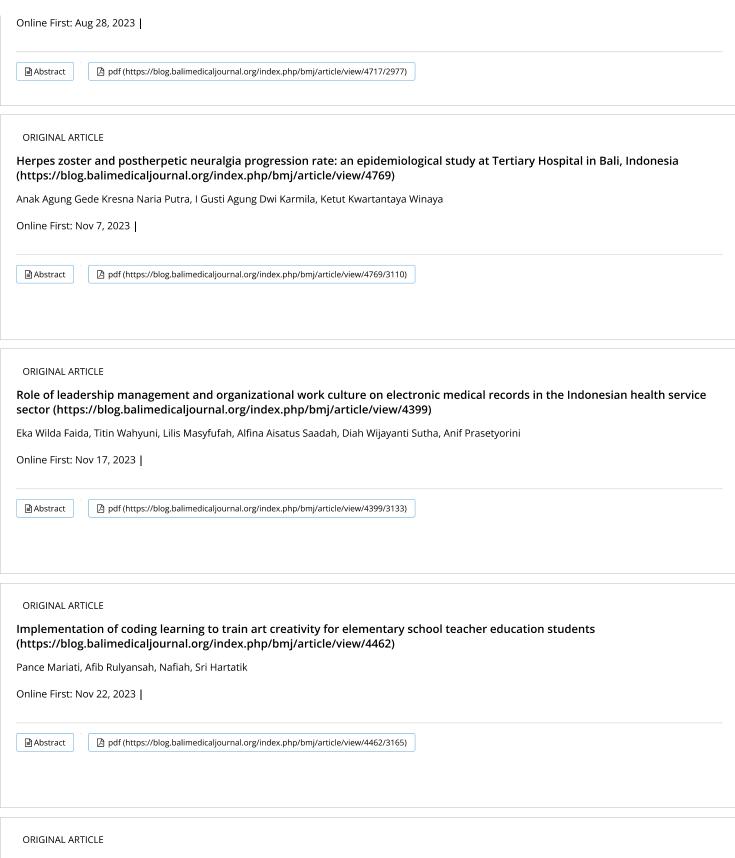


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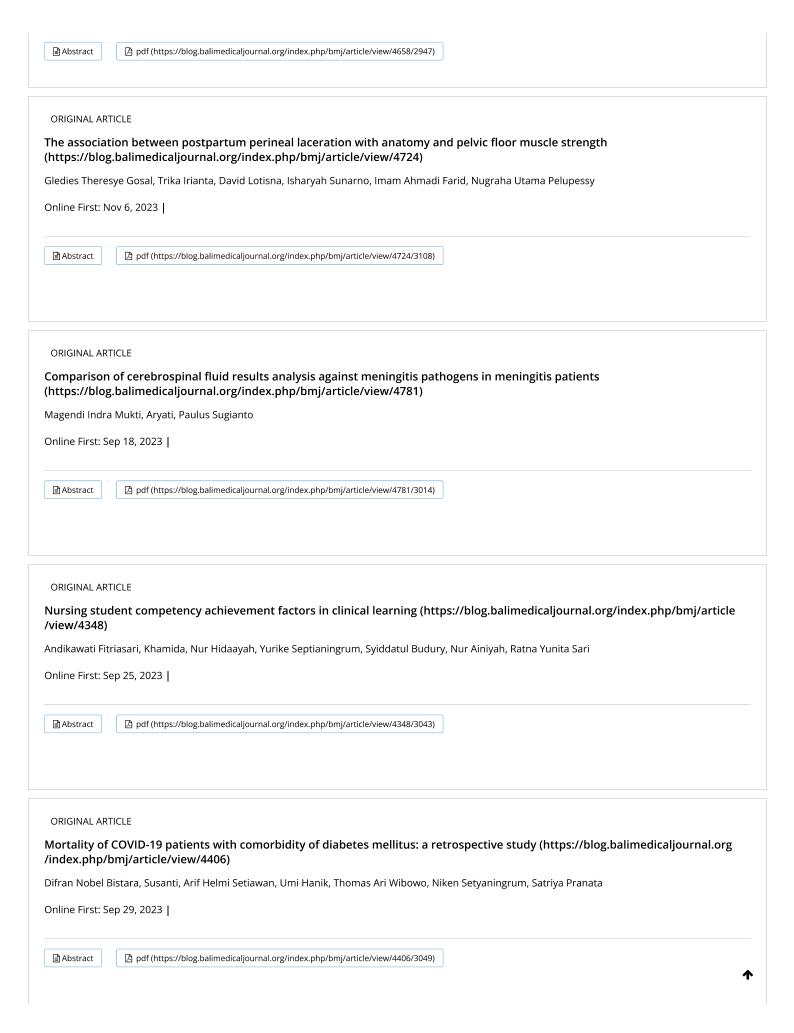




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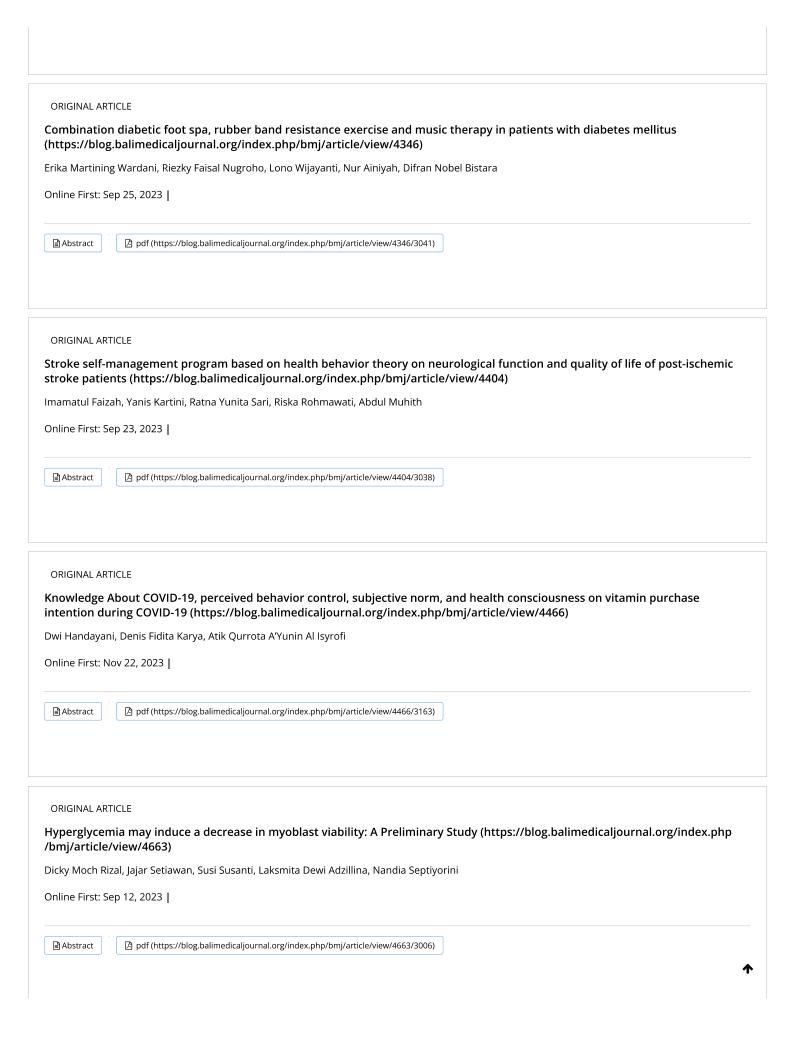
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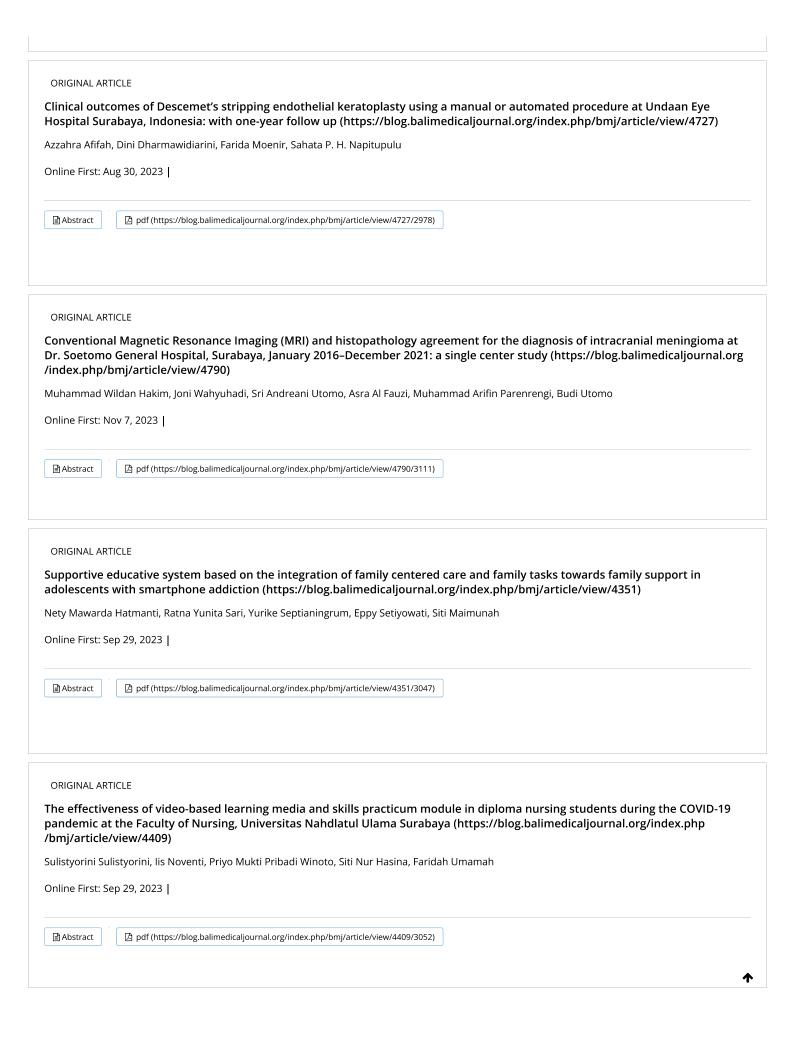
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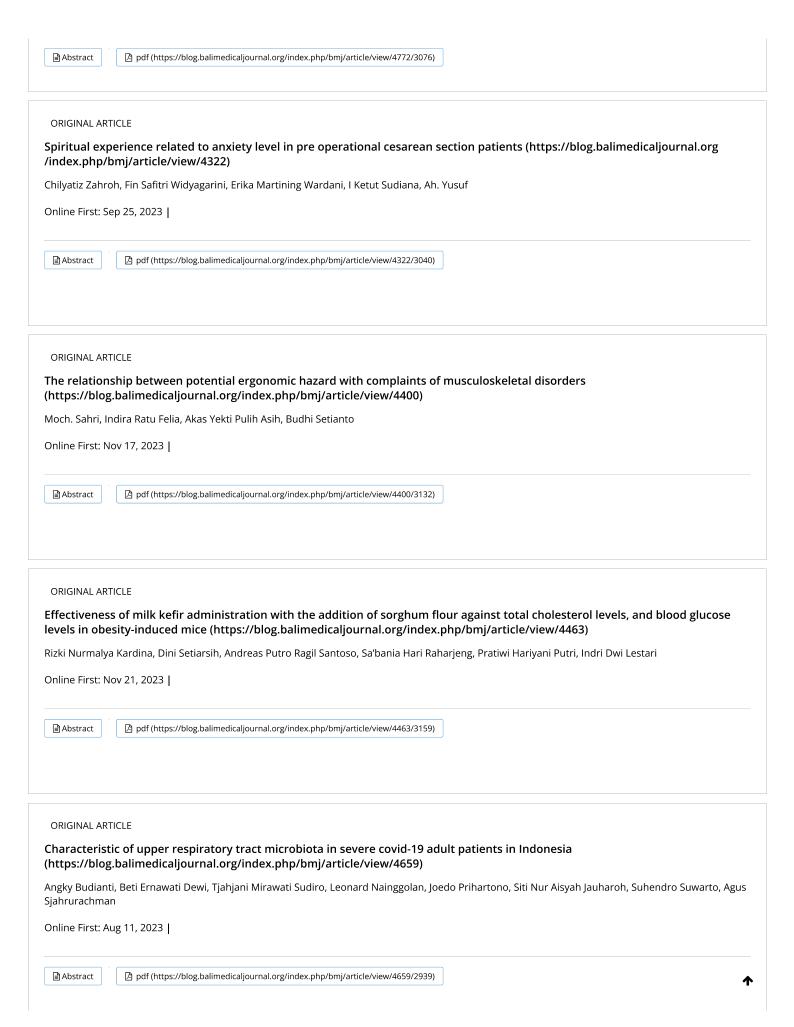
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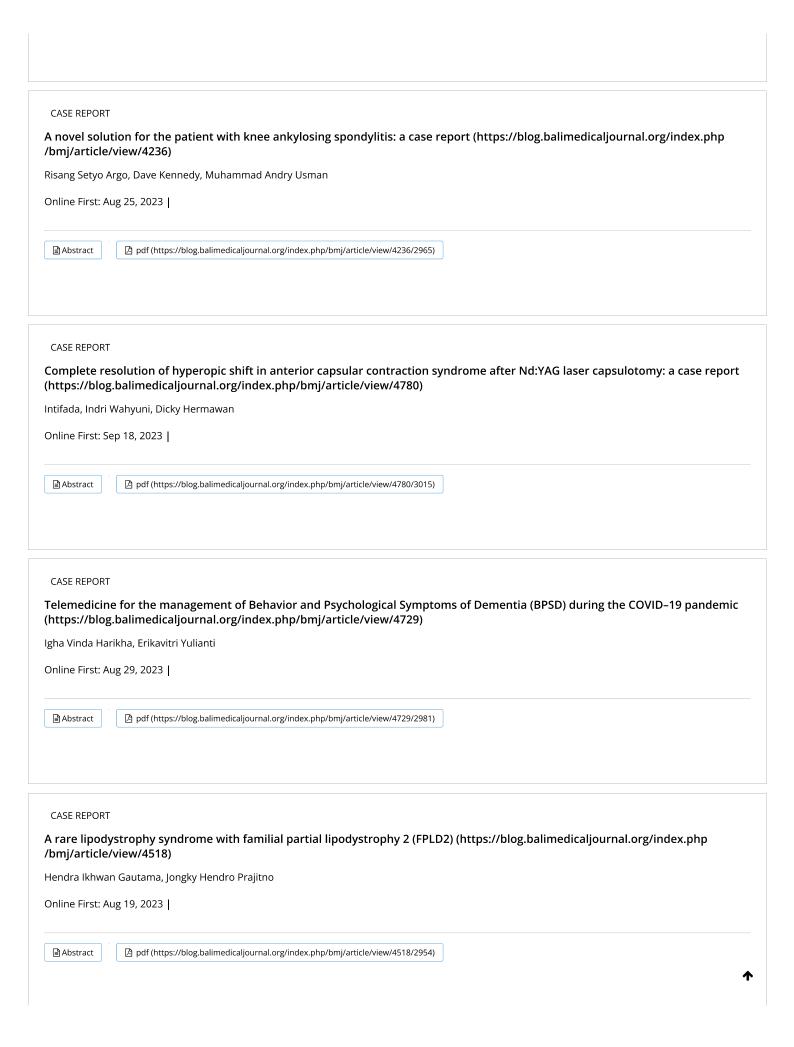
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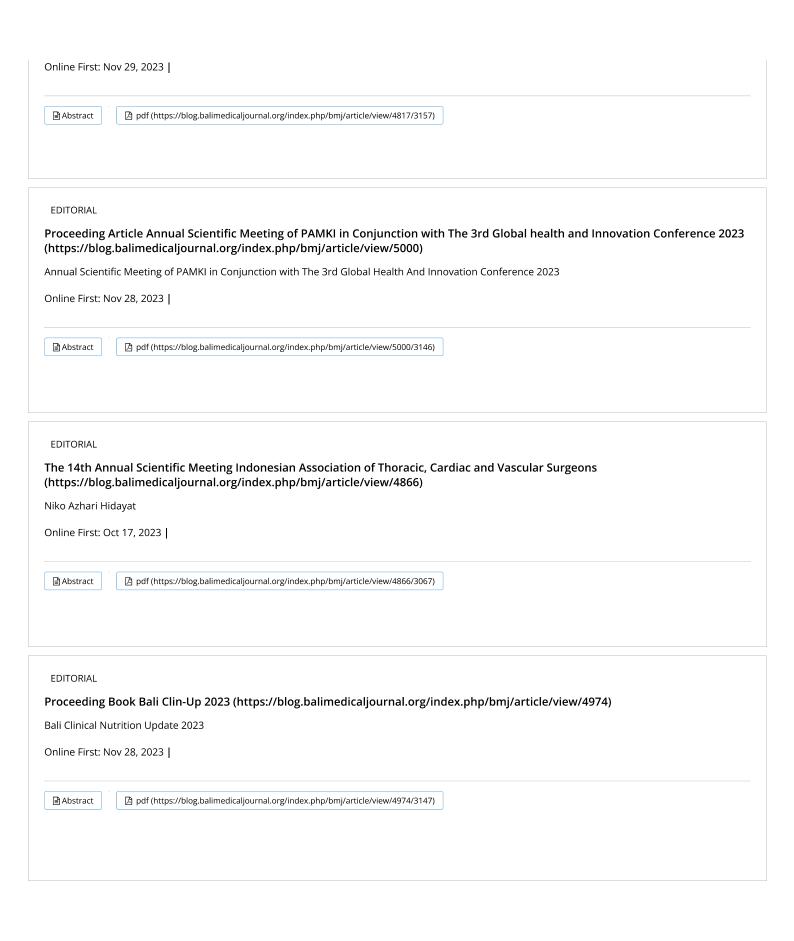
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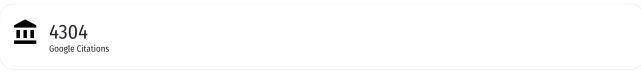
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