

BLACK RICE ETHANOL EXTRACT EFFECT ON ACCUMULATION OF LYMPHOCYTES IN LIPOPOLYSACCHARIDE-INDUCED WISTAR RATS SPLEEN

(EFEK AKUMULASI EKSTRAK ETANOL BERAS HITAM TERHADAP LIMFOSIT PADA LIPOPOLISAKHARIDA YANG DIINDUKSI LIMPUN TIKUS WISTAR)

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ABSTRACT

Infectious diseases are the second leading cause of death in the world after cardiovascular disease. Chronic infections were caused by anaerobic gram-negative bacteria that contain lipopolysaccharides which function as the primary defense of bacteria against antibiotics and antimicrobials. These bacteria can cause chronic inflammation. Chronic inflammation can lead to the accumulation of lymphocytes in the spleen, which plays a role in the immune system. An adequate immune system is needed to overcome these infections through immunostimulators, one of which was obtained from anthocyanins derived from black rice (*Oryza sativa L. indica*). This study aims to determine the effect of black rice ethanol extract on the spleen of Wistar rats induced by lipopolysaccharide. This study is an experimental laboratory study with

a sample of 27 rats given 400 mg/kg BW black rice extract for 14 days; then, rats were induced with LPS 0.3 mg/kg BW intraperitoneally for 24 hours before being CO₂ euthanized, and the spleen was taken to examine the diameter of the lymphoid follicles. The design of this research is the Posttest Only Control Group. The results were analyzed using the One-way ANOVA test with a p-value <0.05, meaning that there was a significant difference in the diameter of the lymphoid follicles between the treatment groups. The results showed that the diameter of the lymphoid follicles in the treatment group was more extensive than that in the positive and negative control groups. In conclusion, the ethanolic extract of black rice can increase the accumulation of lymphocyte cells in the white pulp zone, thereby increasing the diameter of the lymphoid follicles in the spleen of Wistar rats induced by lipopolysaccharide.

Keywords: accumulation; black rice; inflammation; lipopolysaccharide

ABSTRAK

*Penyakit infeksi merupakan penyebab kematian kedua di dunia setelah penyakit kardiovaskular. Infeksi kronis dapat disebabkan oleh bakteri anaerob gram negatif yang mengandung lipopolisakarida yang mempunyai fungsi sebagai pertahanan utama bakteri terhadap antibiotik dan antimikroba. Bakteri tersebut dapat menyebabkan inflamasi kronis. Inflamasi kronis dapat menyebabkan akumulasi limfosit pada organ limpa yang berperan dalam sistem imun. Diperlukan sistem imun yang adekuat untuk mengatasi infeksi tersebut melalui imunostimulator yang salah satunya didapatkan dari antosianin yang berasal dari beras hitam (*Oryza sativa L.indica*). Penelitian ini bertujuan untuk mengetahui pengaruh pemberian ekstrak etanol beras hitam terhadap organ limpa tikus wistar yang diinduksi lipopolisakarida. Penelitian ini merupakan penelitian eksperimental laboratorium dengan jumlah sampel 27 ekor tikus yang diberikan*

ekstrak beras hitam sebanyak 400 mg/kgBB selama 14 hari kemudian tikus diinduksi LPS 0,3 mg/kgBB pada 24 jam secara intraperitoneal sebelum dieutanasia CO₂ dan diambil limpa untuk diperiksa ukuran diameter folikel limfoid. Desain penelitian ini merupakan PostTest Only Control Group. Hasil penelitian dianalisis menggunakan uji One -way ANOVA dengan nilai $p < 0,05$ artinya terdapat perbedaan bermakna diameter folikel limfoid antar kelompok perlakuan. Hasil menunjukkan bahwa diameter folikel limfoid pada kelompok perlakuan lebih besar dibandingkan dengan kelompok kontrol positif dan negatif. Kesimpulannya ekstrak etanol beras hitam dapat meningkatkan akumulasi sel limfosit pada zona pulpa putih sehingga meningkatkan diameter folikel limfoid di organ limpa tikus wistar yang diinduksi lipopolisakarida.

Kata kunci: akumulasi; beras hitam; inflamasi; lipopolisakarida

INTRODUCTION

Infectious diseases are the second leading cause of death in the world after cardiovascular disease, with a percentage of 15.6% in women and 16.7% in men, followed by cancer which has a presentation of 11.8% in women and 13.4% in men.¹ Infectious diseases are a significant health problem in developing countries including Indonesia.² Infectious diseases are divided into two, namely acute infectious diseases and chronic infections.³ One of the leading causes of chronic infectious diseases is gram-negative anaerobic bacteria. These bacteria contain lipopolysaccharide, so they are pathogenic. Lipopolysaccharide is a

central component of the outer membrane of gram-negative bacteria, which serves as the primary defense of bacteria against antibiotics and antimicrobials. Lipopolysaccharide causes inflammation in macrophages and other cells and can cause organ damage if prolonged inflammation occurs. The immune system is needed to prevent and fight infection in the body as a defense against pathogens and reactions to several non-infectious substances, including harmless environmental molecules, tumors, and even unchanged host components. The immune system triggers an inflammatory process to fight infection.^{3,4,5}

Inflammation is a local tissue reaction to infection or injury that involves more mediators than the immune response. Inflammation can cause harm to sufferers due to chemical factors that aim to create a physical barrier against the spread of infection and promote the healing of damaged tissue. The chemicals released (histamine, bradykinin, serotonin, leukotrienes, prostaglandins) can cause reactions in the body such as pain, heat, swelling, increased mucus production, pain, and even organ dysfunction, acute inflammation can occur very quickly and become severe. The inflammatory reaction can stop on its own, responsive to the required therapy, but if therapy fails, a chronic inflammatory process can occur.^{3,6,7} Chronic inflammation is one of the causes of chronic diseases that collectively cause disability and mortality, such as cardiovascular disease, cancer, diabetes mellitus, autoimmune and neurodegenerative. Chronic inflammation can last for months or even years. So that it can cause losses to the body, namely failure to get rid of inflammatory causes, autoimmune responses occur, and persistent low-intensity chronic irritants. Chronic inflammation can lead to the accumulation of mononuclear cells and the proliferation of fibroblasts. Mononuclear cells that accumulate during chronic inflammation

are lymphocytes. The accumulation of lymphocyte cells that play a role in the immune system occurs in the lymphoid organs. The spleen is a peripheral lymphoid organ where lymphocytes participate in the body's immunity. The content of lymphocytes in the spleen organ is one of the most abundant, so a strong enough immune system is needed to prevent diseases caused by chronic inflammatory responses. A strong immune system can be obtained through immunostimulators.^{8,3,9,10,11}

Immunostimulators are molecules that stimulate an immune response; in diseases such as immunodeficiency, immunostimulators can stimulate the development and activity of T cells. Immunostimulators are divided into two types, namely synthetic and natural. Synthetic immunostimulators that are designed and developed with a specific way of working but clinically fail to provide beneficial therapeutic action due to problems with their bioavailability and stability as well as serious side effects, so the authors are encouraged to find out alternative immunostimulators made from other than synthetic, namely natural.⁹ A food ingredient that can be used as a natural immunostimulator is *Oryza sativa L. indica*, a color pigment compound called anthocyanin. Anthocyanin in black rice is

the best content compared to other rice. Anthocyanins are natural color pigments that belong to the flavonoid group. The main anthocyanin in black rice is cyanidin-3-glucoside (C3G), an essential source of anthocyanin in Asia. Anthocyanins as antioxidants have several benefits as protection against inflammation, atherosclerosis, carcinoma, and diabetes. As an antioxidant and anti-inflammatory, black rice contains active phytochemicals such as tocopherols, tocotrienols, oryzanols, B complex vitamins, and phenolic compounds. Based on the bioactive components in anthocyanins and their benefits, it is necessary to know more about the potential of black rice as an immunomodulator. In a 2019 study by Hartanti, Black rice ethanol extract proved to be an immunostimulator by increasing the proliferation of lymphocyte cells in the rat spleen. The method used is lymphocyte isolation and in vitro. In a 2021 study by Hamdalah regarding black rice ethanol extract as an immunostimulator, it was also proven by increasing the germinal center in the KGB of Wistar rats. The effective dose used is 400 mg/. This research was conducted with the hope that black rice ethanol extract could be an immunostimulator and able to reduce the duration of chronic inflammation to prevent the losses that would be caused by chronic

inflammation.^{9,12,13,14,15}

Animals are often used in health research experiments such as feasibility testing, drug substance safety, or disease-related research. White rats (*Rattus norvegicus*) This study used Wistar rats which were induced with lipopolysaccharide. Experimental animals will be injected with lipopolysaccharide, which stimulates an inflammatory process. Based on the description that has been explained, the authors wanted to find out whether the ethanol extract of black rice can be an immunostimulator, namely increasing the accumulation of lymphocyte cells in the spleen organs of Wistar rats induced by lipopolysaccharide using a more straightforward method than previous research, namely, through reading preparations and in vivo.¹⁶

METHOD

This research was conducted at the Biochemistry Laboratory, Faculty of Medicine, Universitas Jenderal Achmad Yani, and the Cytopathology Laboratory, Faculty of Health Science and Technology, Universitas Jenderal Achmad Yani. Ethical approval was obtained from the Padjadjaran University Research Ethics Commission dated 13 October 2020 under number 46/UN6.KEP/EC/2020.

Research Design

This research is an experimental laboratory study with a posttest-only control group design.

Research Subject

The research subjects used in this study were white rats (*Rattus norvegicus*) Wistar strain which had been acclimatized for seven days at the Veterinary Laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani. During the maintenance period, rats were fed pellets, drinking water, and experimental substances according to the treatment group. The rats in this study were divided into three groups: a positive control group, a negative control group, and a treatment group. The standard for experimental animal rooms is a floor area of 20m² in the form of a right-angled rectangle with a height of 2.5-3.0 m. Temperature, relative humidity, and air quality must be maintained to be stable with a temperature of 18-26°C, room ventilation must circulate air 15-20 times every minute, and lighting can be adjusted to bright and dark 12 hours alternately.⁴⁰

Research Object

This study used black rice ethanol extract from the *Oryza sativa L. Indica* species from Garut, which was made at the

Biochemistry Laboratory Faculty of Medicine Unjani.

Research Materials

The materials used for the research were black rice ethanol extract at a dose of 400 mg/kg, rat pellet food and drinking water, 70% alcohol, CO₂ gas, 10% formalin, 1% CMC, lymphocyte cells from the spleen, object glass, cover glass, and Hematoxylin-Eosin dye.

Research Preparation

This study used Wistar rats (*Rattus norvegicus*). The rats used were 27 rats divided into three groups. Preparations made before the study were rats acclimatized in cages for seven days in the Animal Laboratory Room, Faculty of Medicine, Universitas Jenderal Achmad Yani, Cimahi. During the adaptation and research period, the rats were fed pellets and drank sufficiently every day, then the rats' weight was measured.

Preparation of Black Rice Ethanol Extract

The production of black rice ethanol extract was carried out in the biochemistry laboratory of the Faculty of Medicine, Universitas Jenderal Achmad Yani. Black rice is dried and then mashed. After the black rice is refined, it is then

soaked using a 1000ml Erlenmeyer in 95% PA ethanol because anthocyanins can dissolve in polar organic solvents, namely ethanol, with the ratio used between ethanol and black rice being 1:2. After being put into the Erlenmeyer then put into the shaker for 1-2 hours at a speed of 200-250 rpm. Maceration was carried out for three days at room temperature, and after that, it was filtered. The filtrate obtained was then concentrated with a rotary vacuum evaporator at 500°C until a thick filtrate was obtained. The filtrate is then dried using a freeze drier process so that the remaining solvent is lost. The dried filtrate can then be stored in a bottle or cup wrapped in aluminum foil.¹⁵

Treatment Method

Rats were acclimatized for seven days as prepared cages, places to eat, drinking places, feed, and newspaper bedding. This was done to see whether the conditions of the rats matched the study criteria. The standard for experimental animal rooms is a floor area of 20m² in the form of a right-angled rectangle with a height of 2.5-3.0 m. Temperature, relative humidity, and air quality must be maintained to be stable with a temperature of 18-26°C, room ventilation must be able to circulate air 15-20 times every minute, and lighting can be adjusted to bright and

dark 12 hours alternately.⁴⁰ Rats that had been acclimatized were then given black rice ethanol extract for 14 days, which was given once a day in the afternoon, adjusting to rats that are nocturnal animals or active at night. On the 23rd day, LPS was induced at a dose of 400 mg/kg BW. The aim was to determine the effect of black rice ethanol extract on the physiological functions of the body and the behavior of experimental animals. All rats were given fed 25-35g/head, drinking distilled water. Groups 2 and 3 were induced by intraperitoneal LPS. Group 3 was given ethanol extract according to a predetermined dose of 400 mg/kg BW. Rats were sacrificed by inhalation of CO, and termination was carried out for sampling the spleens of white Wistar rats on the 37th day.

LPS Induced Experimental Animal Models

The lipopolysaccharide used comes from *Salmonella typhii*. Injectable lipopolysaccharide was prepared by dissolving LPS in phosphate-buffered saline. Experimental animals on day 23 in the positive control group (K2) and treatment group 1 (P1) were injected intraperitoneally with LPS 0.3 mg/kgBW (ip). LPS 0.3 ml has been dissolved with 0.9% NaCl. The purpose of giving LPS is to induce an immune reaction in the form of an

inflammatory state in rats.

Spleen Preparete Preparations

The spleen was taken and cut transversely, measuring 1x1x1 cm, then immersed in 10% neutral buffered formalin (NBF) solution. Organ samples were then thinned and sliced, stored in tissue gauze, and fixed in an NBF solution. After fixation, use solutions consisting of 70% alcohol, 80% alcohol, 90% alcohol, anhydrous alcohol, toluene, and paraffin wax for dehydration and clarification, gradually over a day. The organ samples were sealed with an embedding device filled with molten paraffin and cooled. To slice cold blocks, use a microtome with a thickness of \pm 4-5 microns. The final process is staining with the Harris hematoxylin-eosin method and mounting media.

Data Analysis

This study used lipopolysaccharide-induced lymphocyte cell accumulation in the rat spleen's rubra pulp. Analysis of numerical data requires a normality test using the Shapiro- Wilk method because the number of samples is less than 50. The results of normal data distribution can be continued with parametric statistical tests using One Way ANOVA. The test can be continued with the

Post Hoc Tukey Test analysis.

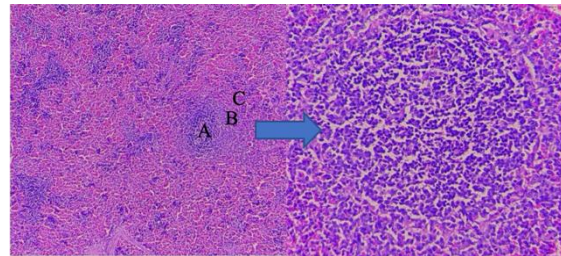
RESULT

Lymphoid Follicle Histopathological

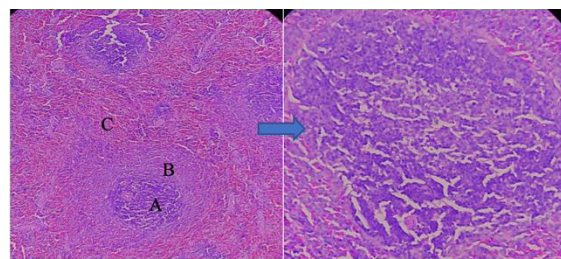
Results

Histopathological preparations were taken from the spleen of male Wistar white rats by staining with Hematoxylin Eosin (HE) to stain the lymphoid follicles of the spleen. Quantitative observations were made with the aim of seeing the size of the diameter of the lymphoid follicles of Wistar white rats.

(a)



(b)



(c)

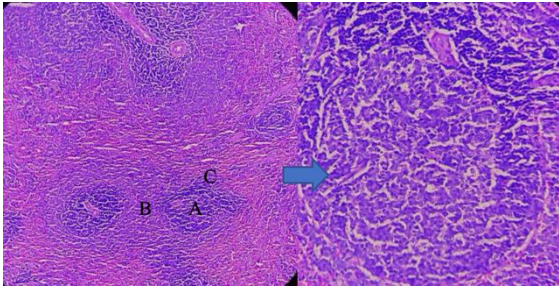


Figure 1. Spleen Histology with Hematoxylin Eosin Staining. The Left Image is 100x Magnification, and the Right Image is 400x. Description (a). Negative Control, (b) Positive Control, (c) 400 mg/kgBW Extract Treatment.

The results of histopathological observations were carried out with an objective magnification of 100x and 400x. In the negative control group (a), the image on the left shows the white pulp area (A), which contains quite dense lymphocytes and other immune cells, with the Germinal Center (B) delimiting the red pulp area (C) and the pulp white (A).

The right image shows the white pulp area (arrow), which contains dense lymphocyte cells but not too tightly, and the Germinal Center area (B) can be seen. In the positive control group (b), the image on the left shows the white pulp (A) with a widened Germinal Center (B) due to stimulation from the white pulp (A) due to an inflammatory process carried by the blood from the red pulp (C).

The image on the right shows a

400x magnification, where you can see the white pulp (arrows) with a lot of necrosis. Results of observations in the 400 mg/kg BW extract treatment group.

The figure on the left shows that the part of the white pulp (A), which contains B lymphocyte cells, is denser than the white pulp in the negative control group and the positive control group. The germinal center (B) in the left image looks enlarged due to the inflammatory response of the white pulp (A).

Histopathological Results of Lymphoid Follicle Diameter Quantitatively

The research results were identified based on the observations that had been made, and the data are presented in Table 1.

Table 1. Wistar rat lymphoid follicle diameter accumulation zone

Group	Lymphoid Follicle Diameter (µm)									Mean ± SD
	1	2	3	4	5	6	7	8	9	
K	0	0	0	0	0	0	0	0	0	0.04
	0	0	0	0	0	0	0	0	0	5 ±
	3	3	4	4	5	4	5	5	4	0.00
N	7	6	1	8	0	8	3	2	3	6
	0	0	0	0	0	0	0	0	0	0.03
	0	0	0	0	0	0	0	0	0	3 ±
P	3	4	3	3	3	3	3	2	2	0.00
	6	1	1	4	2	4	0	8	9	4
	0	0	0	0	0	0	0	0	0	0.04
P	0	0	0	0	0	0	0	0	0	1 ±
	3	4	3	4	4	4	3	4	4	0.00
	9	0	8	0	2	5	6	1	7	3

Based on the data presented in

Table 1, it shows that the mean accumulation zone diameter of lymphoid follicles in Wistar rats with nine repetitions in the negative control group (KN) is 0.045 μm , the positive control group (KP) is 0.033 μm , and the treatment group (P) is 0.041 μm . The largest mean diameter accumulation zone of lymphoid follicles was in the negative control group, and the smallest was in the positive control group.

Table 2. One-Way ANOVA test results in accumulation of wistar rat spleen lymphocytes

Group	Average \pm SD	F (Anova)	p
KN	0.045 \pm 0.006	16.583	0.000
KP	0.033 \pm 0.004		
P	0.041 \pm 0.003		

Description: *One-Way ANOVA* test; $p < 0.05$ (there is a significant difference)
KN = Negative Control; KP = Positive control; P = Treatment.

The research data results were then analyzed using the Shapiro-Wilk normality test. The results of the normal distribution of Wistar rat spleen lymphocyte accumulation normality test showed that data on the accumulation of spleen lymphocyte cells of Wistar rats in the three groups has a normal distribution ($p > 0.05$) so the statistical test used is a parametric test, namely One-Way ANOVA.

The results of the One-Way

ANOVA test in Table 2 show a significant effect between the treatment groups with a significance value of $p = 0.000$ or $p < 0.05$.

Table 3. Tukey's Post-hoc test for Wistar rat spleen lymphocyte accumulation

Treatment (I)	Treatment (J)	p	Conclusion
KN	KP	0.00	Significant
	P	0.131	Not Significant
KP	P	0.003	Significant

Description: *Post-Hoc Tukey* test; $p < 0.05$ (there is a significant difference)
KN = Negative Control; KP = Positive control; P = Treatment.

DISCUSSION

Immunomodulators are all drugs that play a role in improving the immune system by providing stimulation (immunostimulants) to natural and adaptive defense mechanisms or suppressing (immunosuppressants) the immune system that is impaired or has an excessive function. Immunostimulants or immunostimulators increased the immune system's activity to fight infection and disease, which were classified into biological and synthetic immunostimulants. Cytokines, monoclonal antibodies, fungi, and medicinal plants (herbs) are classified as biological immunostimulants, while levamisole, isoprinosine, and muramyl peptidase were classified as synthetic immunostimulants.^{8,7,17}

Immunostimulator acts as an additional treatment for diseases caused by pathogenic microorganisms, relieves infection symptoms, shortens healing time, or are used as a prevention against disease by increasing the body's resistance. Many immunomodulators were found for dietary supplements, especially those made with natural herbal ingredients such as bitter, meniran, noni, and ginger.^{7,18} Black rice (*Oryza sativa L. indica*) is one type of rice that has been consumed for a long time in Southeast Asia. Black rice were consumed as a staple food and an alternative medicine various diseases by kings worldwide, especially in China and Indonesia. It is known as "forbidden rice" because it is black. Black rice, which comes from the black rice plant, is a local variety with the best pigments (especially anthocyanins).

The results of Tukey's Post-Hoc test showed that there was a significant difference between the accumulation of splenic lymphocytes in the negative control and positive control groups ($p=0.000<0.00$) and in the positive control group and the treatment group ($p=0.003<0.00$). Meanwhile, there was no significant difference between the accumulation of spleen lymphocytes in the negative control and treatment groups ($p=0.131>0.00$). It was because the negative control group compared to the positive control group has

a larger diameter compared to the positive control group; the larger diameter in the negative control group is due to the accumulation of lymphocytes and the state of the spleen in the normal rat in general, while in the positive control, the accumulation of lymphocytes in the spleen was not too much, moreover, the extract was not given, and a chronic inflammatory reaction occurred which resulted in Reactive Oxygen Species (ROS) causing tissue damage.¹⁹ In the treatment group, the diameter is also larger than the positive control because the ethanol extract of black rice can act as an immunostimulator so that it can increase the accumulation of lymphocyte cells in the spleen and make the diameter of the follicles larger, besides that the ethanol extract of black rice which contains anthocyanins also has another role. One of them is an antioxidant. Thus, in the treatment group, there was no tissue damage. In the positive control group, anthocyanins had an antioxidant effect that could terminate the propagation chain of free radicals that occurred in the rat's body, and no necrosis occurred in the spleen image.^{20,21,22}

The analysis results show that in the positive control group (KP), LPS can cause necrosis in the white pulp caused when LPS invades the spleen can cause damage to blood vessels. A defense

mechanism occurs when antigens and lymphocytes meet for a long-time, causing cell death due to LPS induced for two weeks. The germinal center area looks widened because it is essential in filtering antigens from LPS to the white pulp; this process takes place continuously. Histology of the spleen in the negative control group showed a normal white pulp containing sufficient lymphocytes and no necrosis in the tissue. The results of the analysis showed that there was no significant difference between the diameter of the lymphoid follicles in the group given black rice ethanol extract for 14 days and given LPS for 14 days with the negative control group (KN), indicating that black rice ethanol extract can be an immunostimulator by increasing lymphocyte cell proliferation and affect the migration of lymphocyte cells in the white pulp. It was due to the presence of flavonoids, one of which is the anthocyanins contained in black rice which can stimulate the work of protein kinase and 5-lipoxygenase enzymes so that IL-2 is produced and activates B cells and T cells to proliferate. So it can be concluded that the ethanol extract of black rice can protect against damage caused by LPS administration.¹²

The histological results of the positive control group (KP), which were only given LPS induction without giving

black rice ethanol extract, showed necrosis in the white pulp zone which was different in the treatment group (KP) black rice ethanol extract affected the lymphocyte cell density in the white pulp where many lymphocyte cells were accumulated in the white pulp. The ethanol extract of black rice contains anthocyanin compounds that act as immunostimulators, thereby increasing lymphocyte proliferation and affecting the migration of lymphocyte cells in the white pulp to become more numerous.

CONCLUSION

Based on the results of this study, black rice ethanol extract could increase the accumulation of lymphocyte cells in the white pulp zone, thereby increasing the diameter of lymphoid follicles in the spleen organs of Wistar rats induced by lipopolysaccharide.

CONFLICT OF INTEREST

There is no conflict of interest in the scientific articles written.

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