The Antioxidant Effect of Indonesian Propolis In Rats Induced by Anthrax Spores

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ABSTRACT

Background: Anthrax is a zoonotic infection caused by the pathogenic *Bacillus anthracis* which causes multi-organ dysfunction and necrosis of the target organ. Anthrax infection causes oxidative stress which is characterized by the formation of ROS that continues to endothelial dysfunction and tissue necrosis. Current management of anthrax using antibiotic arises problems related to the side effects of these antibiotics.

Objective: This study aims to prove the effect of Indonesian propolis as an antioxidant in rats induced by anthrax spores.

Materials and methods: This is a true experimental post-test only control group study on male white rats *Rattus norvegicus*, which were induced with anthrax spore and given ethanolic extracts of propolis (EEP) from Mount Lawu, Indonesia. Antioxidant effect was examined using ELISA on serum SOD as internal antioxidant and ROS level was assessed by serum MDA levels.

Results and Discussion: This study used 40 male rats which were divided into 5 treatment groups. SOD levels decreased in the group without EEP, while the group given EEP before induction showed the highest levels of SOD. Serum MDA levels in the *Bacillus anthracis* spore-induced group showed the highest yield, while the group given EEP and/or without antibiotics showed lower levels. The results of the correlation test for these two variables showed p ≤0.001.

Conclusion: Indonesian propolis has been shown to reduce ROS and increase internal antioxidants in rats induced by *B. anthracis* spore and proven to reduce the level of inflammation in lung tissue.

INTRODUCTION

Anthrax is a zoonotic infection that often appears as an outbreak in an area, with the causative agent being Bacillus anthracis. This infection usually attacks herbivorous animals and can be transmitted to humans, if they have contact with animals or animal products infected with anthrax through the skin, respiratory tract, or digestive tract (1,2). This infection begins with an animal that dies from anthrax, but before the animal dies, the owner usually slaughters the animal and sells it at a more economical price, so that it is widely consumed by the public.

The incidence of anthrax in Indonesia is increasing, in 2007 there was an anthrax outbreak in Pati District, then in 2009 it reappeared in Boyolali District. Anthrax resurged as an outbreak in 2011 - 2015 in Boyolali, Sragen and Pacitan Districts (3). The increased incidence of anthrax cases has made this disease one of the priorities of 14 zoonotic diseases in Indonesia (4).

Transmission that occurs in humans is due to direct or indirect contact with infected animals, which begins with the entry of *B. anthracis* endospores, through skin wounds or along with food or inhalation (2). After the spores enter, they will change into the vegetative form of *B. anthracis*, which then multiplies and can produce toxins. This causes

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an initial response that triggers the expression of proinflammatory cytokines, including Tumor Necrosis Factor Alpha (TNF α), interleukin 1 β (IL-1 β), Interleukin 18 (IL-18) and Interleukin 6 (IL-6), which in turn can causes the production of reactive oxygen species (ROS), which is assessed by levels of Malondialdehyde (MDA) in serum (5, 6). If the production of ROS is excessive, it will cause endothelial dysfunction, and necrosis (5).

Update management to prevent dysfunction in the target organs of exposed individuals are currently using antibiotics after symptoms and signs of anthrax appear (2). There are several things that often occur with the use of this antibiotic, including side effects such as nausea, vomiting, and the emergence of resistance. Therefore, it is necessary to make other efforts to reduce this, namely with natural ingredients, which are easy to obtain, have no side effects and do not cause toxic effects, such as propolis. Propolis is a product of bees with several components that affect biological activity (7). Propolis from the slopes of Mount Lawu in the form ethanolic extracts of propolis (EEP) is a local product with the active ingredient Caffeic Acid Phenethyl Ester (CAPE), which in several previous studies had an anti-inflammatory effect on bacterial or viral infections and antioxidants (8). This propolis from Mount Lawu has a high CAPE content of 30.24 ± 3.53 x 106 gr and a quercetin content of $4.42 \pm 0.50 \times 10$ -6 gr (9). The results of a study by Diding (2013), show that propolis isolate has the potential as an antioxidant at a dose of 200 mg / kg body weight every day for 30 days, which is proven to reduce MDA levels and can improve wounds in the diabetic feet of Balb / C mice (10).

This study aims to prove the antioxidant effect of Indonesian propolis in mice induced by anthrax spores, with measuring ROS production using MDA levels in serum and assessing serum superoxide dismutase (SOD) levels as internal antioxidant in preventing lung tissue damage.

MATERIALS AND METHODS

This study is a true experimental post-test only control group design, using male rats *Rattus norvegicus* as experimental animals because this mouse has Anthrax toxin receptor 2 (ANTXR2) which is very adaptable and this mouse is easy to find and has produced many hereditary strains, which are used for a variety of purposes, including medical testing and behavioral studies (11, 12).

Research was carried out in three places, Indonesian Research Center for Veterinary Science (BALITVET) Bogor, the Experimental Animal Center for the Inter-University Study Center (PAU) of Gajah Mada University, Yogyakarta and the Pathology Anatomy, Faculty of Medicine, Sebelas Maret University, Surakarta, Indonesia.

Preparations of ethanolic extracts of propolis (EEP)

Preparations propolis extract follows: 10 g of pulverized sample was weighed and dissolved in 100 mL of 70% ethanolic solution in a 1:10 (w/v) ratio, while another 10 g of pulverized sample was weighed and dissolved in 50 mL of 70% ethanolic solution in a 1:5 (w/v) ratio. Next, samples were shaken (200 rpm) at 28 °C for 1 or 7 days. To prevent excessive heating the samples were immediately placed in ice and water baths. Samples were stored at 4 °C. in dark containers.

Preparations of anthrax animal models

The animals were anaesthetized using ketamine (24 mg/kg BW, intramuscular injection), then waited 2 minutes, the hairs on the rats' backs were shaved in a rectangular shape. After that, disinfect with 70% alcohol and determine the injection site. Dry B. anthracis (BCC 602) spores in the media were then diluted with 10 ml of sterile aqubides and taken with a 1 cc syringe, as much as 1 cc. Liquid spores were injected by subcutan injection point as much as 0.2 cc, which is equivalent to 2×10^{11} CFU of spores. The rats were then returned to their cages and evaluated every day for up to 14 days.

Animals

Rattus norvegicus rats (weighing 180-200 g) were purchased from the Central Animal House, Department of

Experimental Medicine, Inter-University Study Center (PAU) of Gajah Mada University, Yogyakarta, and maintained in an air-conditioned room $(25 \pm 1 \text{ °C})$ with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum*. The experimental study was approved by the Ethical Committee of Sebelas Maret University (Reg No.015/UN27.06.6.1/ KEPK/EC/2020), Surakarta, Indonesia.

Experimental design

The rats were randomly divided into five groups, with 40 *Rattus norvegicus*, aged 3-4 months and weighing 180-200 grams, were divided into five groups, each containing 8 rats. First group (P1) is rats induced by anthrax spores, are given EEP 200 mg / kg bw seven days before the induction of anthrax spores, are given EEP 200 mg / kg bw for up to 14 days, second group (P2) is rats induced by anthrax spores and given EEP 200 mg / bw for 14 days, third group (P3) is rats induced by anthrax spores and given EEP 200 mg / bw for 7 days, fours group (P4) is rats induced by anthrax spores and given Amoxicillin 9 mg / kg bw and EEP 200 mg / kg bw for 14 days, and five group (K) is rats induced by anthrax spores as control. White rats were sacrificed 14 days after subcutaneous injection of B. anthracis spores 2 x 10¹¹ CFU. After 14 days, the animals were anaesthetized using ketamine (24 mg/kg BW, intramuscular injection) and sacrificed by cervical dislocation.

Biochemical analysis

Blood was collected in tubes with a mixture of EDTA for the SOD serum. Lung tissue was taken and made as a histological preparation, for histopathological examination. Serum collection for the examination of MDA was examined using the thiobarbituric acid reactive substances (TBARS) method and SOD was using examined ELISA method.

Lung Histopathology analysis

Prepare a container of 10% neutral buffered formalin, and label the container. All lung lobes and trachea is then placed in formalin. Lungs should be fixed for at least 24 h, although longer times may be required, HE stains.

Statistical analysis

Statistical evaluation was performed using a one-way analysis of variance (ANOVA), using the statistical package of social science (SPSS) version 22.0. The significance level was set at p < 0.05.

RESULTS AND DISCUSSION

Results

In this study, the MDA level was a parameter used in assessing oxidative stress. The data normality test with the Shapiro Wilk test showed that MDA levels in all groups were normally distributed and would be followed by the ANOVA test.

Group	N	means ± SD	р	
P1	8	1.893 ± 0.188		
Р2	8	2.282 ± 0.133		
Р3	8	5.046 ± 0.344	0.001	
P4	8	2.717 ± 0.383		
К	8	9.642 ± 0.279		

Table 1: Means and Standard Deviation of MDA levels

Table 1 shows the highest average MDA in the control group, namely $9,642 \pm 0.279$ nmol / ml, while the lowest group is in P1, namely $1,893 \pm 0.188$ nmol / ml. The mean of the P2 group was almost the same as the P4 group, being

2,282 \pm 0.133 nmol / ml and 2,717 \pm 0.383 nmol / ml, respectively. The ANOVA test results showed a value of p<0.001, meaning that there were significant differences between these groups.



Figure 1: ANOVA and Post Hoc Tuckey Results of Serum MDA Levels

Figure 1 shows a significant difference in serum MDA levels between the control group against the P1 group with $p \le 0.001$, the control and P2 group with $p \le 0.001$, the control group and P3 group with $p \le 0.001$, and the control group and P4 group with $p \le 0.001$.

Antioxidant production level was measured using serum SOD levels. The data normality test with the Shapiro Wilk test showed that SOD levels in all groups were normally distributed and would be followed by the ANOVA test.

Table 2: Means and Standard Deviation of SOD levels

Group	Ν	means ± SD	р
P1	8	79.715 ± 4.377	
P2	8	69.261 ± 4.615	
Р3	8	48.565 ± 6.899	0.001
P4	8	59.838 ± 3.392	
К	8	23.565 ± 4.105	

Table 2 shows the highest mean of SOD levels in the P1 group, namely $79,715 \pm 4,377\%$ while the lowest mark was in control group with $29,563 \pm 4,105\%$. The ANOVA

test results showed a value of $p \le 0.001$, meaning that there were significant differences between these groups.



Figure 2: ANOVA and Post Hoc Tuckey Results of Serum SOD Levels Figure 2 shows a significant difference in serum SOD levels between the control group against each other group with p≤0.001.

Group Inflammation Score of Lung Tissue (%)							
(n = 8)	Skor 0	Skor 1	Skor 2	Skor 3	Total		
К	0	4	4	0	8		
	(0%)	(50%)	(50%)	(0%)	(100%)		
P1	0	0	6	2	8		
	(0%)	(0%)	(75%)	(25%)	(100%)		
P2	0	0	7	1	8		
	(0%)	(0%)	(87.5%)	(12.5%)	(100%)		
P3	0	1	7	0	8		
	(0%)	(12.5%)	(87.5%)	(0%)	(100%)		
P4	0	1	6	1	8		
	(0%)	(12.5%)	(75%)	(12.5%)	(100%)		

Score 0 : negative

Score 1 : mild inflammation of the peribronchial and interstitial tissues of the lung.

Score 2 : moderate inflammation of the peribronchial and interstitial tissues of the lung.

Score 3 : severe inflammation of the peribronchial and interstitial tissues of the lung with necrotic tissue and inflammation of the suppurative granulomatosa.

Table 3 shows the control group with a score of 1 and 2 of 50%, no lung tissue with a score of 3. Group P2 got 25% with a score of 3 and 75% with a score of 2. Groups P2 and P3 got 87.5% with a score of 2, while group P4 75% with a score of 2.



Figure 3 : Histopathological picture of lung tissue (magnification 400 x). Yellow arrows indicate areas of inflammation.

The control group showed severe inflammatory cells accompanied by necrotic tissue (A), group P1 showed moderate category inflammatory cells (B), group P2 showed moderate inflammatory cells with a score of 2 (C), group P3 showed moderate category inflammatory cells (D) and group P4 shows mild inflammation of the peribronchial and interstitial tissues of the lung.



Figure 4: ANOVA and Post Hoc Tuckey Results of Histophatology lung tissue

Figure 4 shows a significant difference in inflammation lung levels between the control group against each other group with $p \le 0.05$ in groups P4.

DISCUSSION

Free radicals trigger peroxidation of cell membrane lipid layers and will produce MDA compounds. In the process of infection, the presence of an incoming infectious agent, for example the induction of anthrax spores, will trigger an innate response from the immune system that stimulates the formation of ROS (13). The presence of TNF α expression can also lead to the formation of ROS (14). A high increase in ROS in the body can increase the inflammatory response which results in tissue necrosis (15), so that lowering ROS can prevent apoptosis and necrosis.

In this study, MDA levels in the group with induction of anthrax spores were very high at $9,642 \pm 0.279$, compared to the treatment group. These results are in accordance with the research of Agarwal et al., (2017) which states that the presence of infection causes an increase in oxidative stress, which can be assessed by examining serum MDA (16).

It was shown during our experiment that the administration of EEP at a dose of 200 mg / kg bw seven days before the induction of B. anthracis spores had the best effect to reduce the mean serum MDA level at 1,893 ± 0.188, compared to EEP administration immediately after induction. Giving only EEP just after the induction of B. anthracis spores for 14 days, showed a better effect of reducing serum MDA levels at the point 2.282 ± 0.133, compared to the combination of EEP with standard antibiotics for 14 days, at 2.717 ± 0.383, and the group only given EEP for 7 days, namely 5.046 ± 0.344. These results are in accordance with research conducted by Diding et al., (2013) which shows that a dose of 200 mg / kg bw / day for 30 days can reduce serum MDA levels, increase levels of soluble Receptors for Advanced Glycation End Products (sRAGE), and the presence of wound repair in diabetic foot model Balb / C mice induced by streptozotocin (10).

EEP administration for 14 days gave better results than EEP administration for 7 days *after B. anthracis* spore induction, with $p \le 0.01$. The results are similar with the study of Koksel et al., 2005, which stated a decrease in serum MDA levels in mice induced with gram-negative LPS bacteria by intraperitoneal injection with caffeic acid phenethyl ester administration (10, 17).

The induction of anthrax spores caused a decrease in serum SOD levels, namely 79.715 \pm 4.377. This is consistent with research Karadas, (2016) in patients with cutaneous anthrax showing an increase in MDA levels and a decrease in SOD levels (18). The comparison between the control group and the group given EEP was statistically significant p <0.001.

Based on the results of the histopathological examination of the alveolar and lung parenchymal tissue, it showed that each 50% of the samples showed mild to moderate inflammation in the peribronchial and pulmonary interstitial tissues, and the highest mean rank was in the group of rats induced with B. anthracis spores, namely 25.75. These results are consistent with the study of Koksel et al. (2006), namely the presence of necrosis in lung tissue in mice induced with gram-negative LPS through intraperitoneal injection (19). Giving EEP at a dose of 200 mg / kg bw, has an effect on improving the level of inflammation in the lung tissue, especially those given together with standard antibiotics, namely 23.565 \pm 4.105. The results of this study indicate that the administration of EEP at a dose of 200 mg / kg bw can reduce serum MDA levels and increase serum SOD levels, as an internal antioxidant, so as to prevent necrosis in the lung tissue of rats induced by anthrax spores.

CONCLUSION

Indonesian propolis has been shown to reduce ROS and increase internal antioxidants in rats induced by *B. anthracis* spore and proven lung tissue damage.

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