

RESEARCH ARTICLE

Indonesian Propolis Inhibit Proinflammatory Cytokines, Apoptosis and Oxidative Stress in Anthrax Animal Model

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Abstract

BACKGROUND: Anthrax is a zoonotic disease caused by *Bacillus anthracis*, whose endospores stimulate the release of pro-inflammatory cytokines and promote oxidative stress. Propolis, a natural resource that can be found in Indonesia, has been proven to have anti-apoptotic and antioxidant role which might be a potential adjuvant therapy for anthrax treatment. Hence in this study we aimed to investigate effect of propolis as an anti-inflammatory, anti-apoptotic, and antioxidant in anthrax rats model by examining the level of tumor necrosis factor (TNF)- α , caspase-3, and malondialdehyde (MDA), respectively.

METHODS: This was an experimental post-test only study with 40 male rats weighed 180-200 g that were induced by anthrax spores injected subcutaneously. The rats were divided into one control positive group and four intervention groups that were administered with 200 mg/kgBW propolis extract for 7 to 14 days. The levels of serum TNF- α , caspase-3, and MDA were measured using enzyme-

linked immunosorbent assay (ELISA) and analyzed with bivariate analysis.

RESULTS: TNF- α , caspase-3, and MDA level were found lower in anthrax rats model given ethanol extract of propolis than the control group. The lowest concentration of TNF- α value was found in group administered with propolis extract 200 mg/kgBW for 7 days before the anthrax induction (6.136 \pm 0.205 pg/mL). The results were similar to the MDA serum and caspase-3 which were the lowest when the propolis was administered 7 days earlier (1.893 \pm 0.188 nmol/mL and 2.040 \pm 0.067 ng/mL). There was significant difference in the TNF- α , caspase-3, and MDA serum levels ($p \leq 0.001$) compared to control group.

CONCLUSION: Propolis has anti-apoptotic and antioxidant effects which can be used as complementary therapy in anthrax infection.

KEYWORDS: propolis, anthrax, anti-apoptotic, antioxidants

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Introduction

Anthrax is one of zoonotic disease which is caused by gram-positive bacteria *Bacillus anthracis*.(1) Previous research showed 5,197 incidences of anthrax in humans between 2009 and 2013, with 86 (1.7%) of those cases of human

death due to anthrax occurring in Ethiopia.(2) The increasing incidences of anthrax in Indonesia began in the early 2020. An epidemic occurred in Gunung Kidul, Yogyakarta, where approximately 600 people were infected due to consuming anthrax-infected animal meat and 15 people showed clinical manifestations. Anthrax disease had become one of the zoonotic disease priorities in

Indonesia due to the increased incidence of anthrax cases.(3)

Anthrax is transmitted to human transmission by direct or indirect contact with infected animals. *B. anthracis* endospores entered the circulating blood via skin wounds or along with ingested or inhaled food.(4) The vegetative forms of endospores would replicate and produce toxins that induce initial response to elicited the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-1 β and also lead to reactive oxygen species (ROS) release.(5,6) Pro-inflammatory cytokines and ROS would induced apoptosis which is characterized by an increase of caspase-3, and dysfunction of multiple organs, such as skin, lung, kidney and liver.(7-9)

Antibiotics regimen had became the current strategies to treat and prevent complications in the affected individual's target organs.(4) Adverse effects and the development of bacterial resistance became several considerations to the need of alternative regimen of therapy. Natural products has been used to overcome antibiotic resistance. Natural sources contain antioxidants and antibacterial agents such as polyphenols, vitamins, and carotenoids are the dominantly involved in boosting the defense system of organisms. (10) Propolis, a natural product, has been widely used in traditional medicine and shown to have various biological activities, including antiapoptotic, anti-oxidative, anti-inflammatory, antimicrobial, antiprotozoal, anti-parasitic, anti-ulcerative, anti-tumor, antiviral, and hepatoprotective activities, but scientific research about antioxidants activities on propolis in Indonesia is still limited.(11-12) Propolis production in Indonesia from conventional nest is about 18.5 g/colony/year. The superiority of Indonesian propolis is that its caffeic acid phenethyl ester (CAPE) levels are the highest compared to other regions.(13) Propolis from the slopes of Mount Lawu in the form of ethanol extract propolis (EEP) is one of the local products with the active ingredient, CAPE, which in several previous studies has an anti-inflammatory effect on bacterial or viral infections and antioxidants. Indonesian propolis has a high CAPE content of $30.24 \pm 3.53 \times 10^{-6}$ g and quercetin content of $4.42 \pm 0.50 \times 10^{-6}$ g.(14,15)

The current management in preventing organ dysfunction in exposed individuals, is to use prophylactic antibiotics with ciprofloxacin for 60 days and administration of penicillin or macrolide antibiotics as curative therapy after clinical manifestations of anthrax appear. The use of antibiotics is often a problem, because of the side effects in the form of allergic reactions, nausea, vomiting, and the

emergence of antibiotic resistance. Therefore, it is necessary to make other efforts to reduce this problem, namely by using other regimens derived from natural ingredients, easy to obtain and without side effects, namely propolis.

Ethanol extract of various herbs, such as those extracted from *Cosmos caudatus*, mangosteen pericarp, and *Piperomia pellucida* L., has been reported to affect inflammatory cytokine also play a role as natural antioxidant in inflammation pathway.(16,17) CAPE, is an ethanol extract of propolis, has been shown to have anti-apoptotic effects and antioxidants.(12) In the previous study, the benefit of ethanol extract was proved to prevent skin manifestations and tissue necrosis in anthrax rats model. Meanwhile, this study objects to investigate more systemic effect of ethanol extract of propolis in anthrax.(14) The limited of research on propolis in Indonesia underlies us to conduct research on propolis. Thus, in this paper we aim to analyze propolis in extract form as anti-apoptotic and antioxidant to prevent organ dysfunction in individuals exposed to anthrax.

Methods

Preparation of *B. anthracis* Spores

The *B. anthracis* isolated in frozen-dried form was dissolved by using sterile saline phosphate buffer or 0.9% NaCl, then inoculated on blood agar media and cultured for 24 hours at 37°C. The culture was then harvested, suspended, and evaluated for purity using 0.9% NaCl. A 0.2 mL suspension of *B. anthracis* was inoculated on spore media, incubated at 37°C for 72 hours (when 90% of the spores had developed), and then re-incubated at room temperature for 3 days. To harvest the spores, 10 mL physiological NaCl was put in to the tube and collected. After checking for purity, the spore suspension was heated to 65°C for 1 hour to eliminate vegetative cells. The spore suspension's level was subsequently calculated to be 10^8 colony forming unit (CFU)/mL (cultured spores).(18-20)

Preparation of Propolis Extracts

The dried propolis was collected from the bee keepers at the Lawu Mountain region, Central Java. The production of propolis ethanol extract included combining the 0.1 g of dried propolis with 30 milliliters of water. Then begin the 12-hour maceration procedure. The specimen was put in a shaker and centrifuged for 30 minutes at 30°C at a speed of 50 rpm. The recovered supernatant was filtered 5 hours later. The same process was subsequently carried out five

times more. The soaking duration was increased to 24 hours, and the stirring operation was done without the aid of a shaker.(21,22)

Caffeic Acid Phenethyl Ester (CAPE) levels was measured using a double-beam UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), according to the Prussian blue method. The purpose of measuring CAPE was to select the highest level of CAPE in Indonesia. Previous study showed that propolis had the potential as an antioxidant at a dose of 200 mg/kgBW used for 30 days, proved to be able to reduce MDA levels and played roles on wound repairment on the diabetic feet of Balb/C mice.(23,24)

The CAPE standard (284.31 g/mol) was determined by dividing the standard CAPE into six size groups namely 0, 4, 8, 12, 16, and 20 μ L. Each standard CAPE size group was added with 400 μ L K₃Fe (CN) 6 0.0008 M/0.1 M HCl and 400 μ L FeCl₃ 0.1 M/0.1 M HCl. After it was shaken and waited for 7 minutes, then the absorbance of color on a spectrophotometer observed with a wavelength of 700 nm. The absorbance value is recorded then the data is converted into a standard CAPE graph, where the x-axis shows the standard CAPE value and the y-axis shows the absorbance value.(25)

Experimental Animals

Male rats (*Rattus norvegicus*) weighing roughly 180-200 g were acquired from the Veterinary Faculty of Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats were fed with standard BR-I rat food with an amount adjusted to their average body weight. The animals' housing was kept at a constant temperature of 25-28°C. One week prior to the experiment, the rats were acclimated to their new surroundings. Male *Rattus norvegicus* were chosen as experimental animals because of their genetic closeness to humans and their capacity to adapt to the laboratory setting.(26) The inclusion criteria were healthy male rats with sparkling eyes, non-dull hair, activity and a decent appetite, aged 3-4 months, and weighing 180-200 g, While the exclusion criteria were sick male white rats with non-glowing eyes, dull hair, inactivity, unwillingness to feed, and weight loss. To reduce possible confounders, the confounding variable from mouse characteristics, strain, cage temperature, blood sample, food, humidity, and lighting was controlled in this study.

The experimental animals in this study were treated in accordance with the 3R principle, as defined by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs), and received approval from The Health Research Ethics Committee of Sebelas

Maret University, Surakarta (No. 015/UN27.06.6.1/KEPK/EC/2020).

Anthrax Induction

Anthrax rats were induced by subcutaneous injection of anthrax spores that has been incubated, cultured and harvested before.(26) The rats were anesthetized with 75-100 mg/kg BW of ketamine injection and the hair on the rats' backs was shaved in a rectangular shape. Then, the shaved area was disinfected with 70% alcohol and the injection sited was decided. The dried form of *B. anthracis* spores in the media was diluted with 10 mL sterilized water and taken with 1 mL syringe. The liquid form of *B. anthracis* spores injected subcutaneously into the injection site around 0.2 mL or 2 x 10¹¹ CFU Spores. Rats were put back to the cage and evaluated for 14 days.(27)

Experimental Design

This was a randomized controlled trial with only a post-test only control group. The male rats were separated into five groups, namely one control positive group and four interventional groups, and each group consist of eight rats. Rats in the control group (C) were injected by anthrax spore only without propolis administration; rats in the intervention group 1 (I1) were given propolis 200 mg/kg BW for 7 days before being inoculated with anthrax spores for up to 14 days; rats in the intervention group 2 (I2) were given anthrax spores and 200 mg/kg BW propolis single dose; rats in the intervention group 3 (I3) were given anthrax spores and propolis 200 mg/kg BW for 7 days; rats in intervention group 4 (I4) received anthrax spores, amoxicillin 9 mg/kg BW, and propolis 200 mg/kg BW for 14 days (Figure 1). According to our preliminary studies, the dosage of propolis 200 mg/kg BW demonstrated significant antioxidant effect. As for the duration was also based on our prior studies.(14)

Detection of TNF- α , Caspase-3, and MDA

Serum collection for TNF- α , caspase-3, and MDA levels was performed at the end of the study and analyzed by Enzyme Linked Immunosorbent Assay (ELISA). First, serum was collected from all subjects, then the serum samples were centrifuge at 1,500 rpm for 15 min and stored in a temperature of -60°C. Measurement of serum MDA levels is carried out by spectrophotometric methods. The working principle was to use the NWK-MDA01 assay reaction, based on the reaction of MDA with Thiobarbituric Acid (TBA) absorption which is read with a wavelength of 532 nm. Malondialdehyde was measured using concentrations of Thiobarbituric Acid Reactive Substances (TBARS).

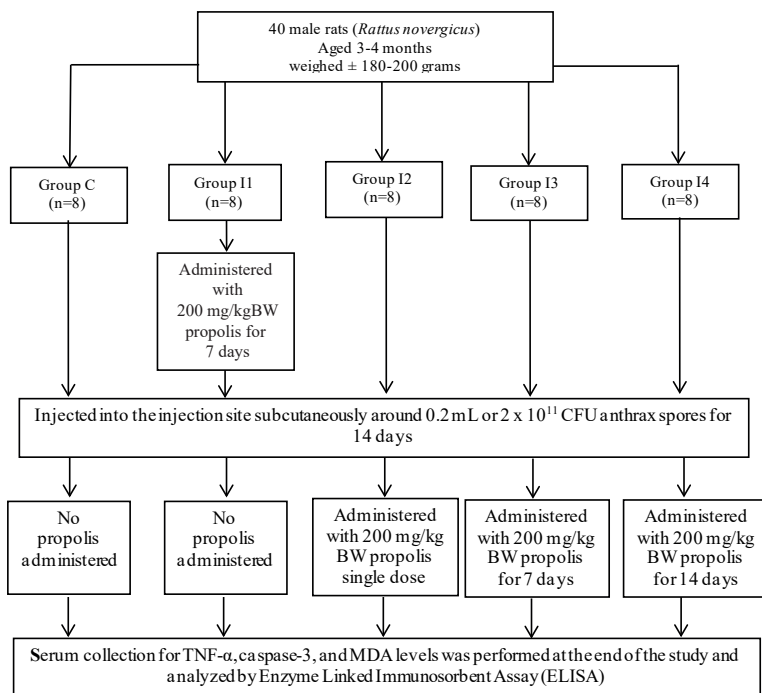


Figure 1. Study group and experimental design. Total 40 rats were divided into 1 control group and 4 treatment groups.

After the reagent and sample was prepared, 100 μ L of serum was added into the sterile test-tube and incubated for 2.5 hours at 4°C. Then, a 100 μ L of biotin antibody, streptavidin, and TMB One-Step Substrate Reagent added into each tubes and incubated for 1 hours, 45 mins, and 30 mins, respectively. Reading was done at 450 nm. The ELISA Kits used for this study are RayBio® Rat TNF alpha ELISA Kit, RayBio® Rat Caspase-3 ELISA Kit, and RayBio® Rat MDA (Norcross, GA, USA).

Statistical Analysis

The obtained data including levels of TNF- α , caspase-3, and MDA was analyzed using SPSS for Windows Release version 23.0 (IBM Corporation, Armonk, NY, USA). Categorical data were analyzed with the non-parametric test (Kruskal Wallis and Mann-Whitney). Numerical data obtained after meeting the criteria were tested using the analysis of variance (ANOVA) test and T-test. The $p < 0.05$ was considered statistically significant.

Results

TNF- α levels were shown in Figure 2. Group C had the highest TNF- α levels after 14 days (14.247 ± 0.311 pg/mL), whereas group I1, that was given propolis 200 mg/kg BW for 7 days before being inoculated with anthrax spores, had the lowest TNF- α concentration (6.136 ± 0.205 pg/mL). According to the post-hoc tuckey analysis, group C had significant difference with group I1, I2, I3, and I4 ($p < 0.05$),

which means that there were significant differences between group.

Furthermore, as for the highest mean serum MDA level was found in the group C (9.642 ± 0.279 nmol/mL), meanwhile group I1 recorded the minimum MDA level (1.893 ± 0.188 nmol/mL). The mean serum MDA level was measured nearly similar between group I3 and I4, 2.282 ± 0.133 nmol/mL and 2.717 ± 0.383 nmol/mL, respectively. The statistical analysis showed that administration of propolis 200 mg/kg BW for 7 days before being inoculated with anthrax spores for up to 14 days had significant difference to decrease serum MDA concentration in anthrax rats ($p < 0.001$) (Figure 3).

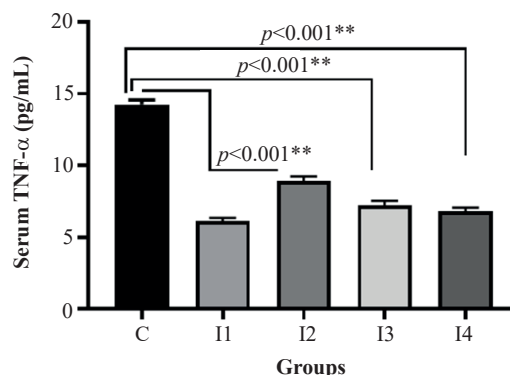


Figure 2. Serum TNF- α levels in anthrax rats administered with propolis extract. There was a significant difference between groups after tested with ANOVA test. Post-Hoc Tuckey Test also showed significant result, except between group I3 and I4. ** $p < 0.001$ and * $p < 0.05$ were considered significant.

It shows that caspase-3 levels in the group C was the highest after 14 days (7.226 ± 0.168 pg/mL), whereas caspase-3 levels in group I1 was the lowest (2.040 ± 0.067 pg/mL). According to the post-hoc test, group C has significant association with I1, I2, I3, and I4 ($p < 0.05$). When compared to the group that received propolis for 7 days (5.363 ± 0.652 pg/mL), the 14-day group had lower caspase-3 levels (3.366 ± 0.272 pg/mL).

Discussion

This study found that the administration of propolis extract 200 mg/kgBW in anthrax rats model decreased the level of TNF- α , MDA, and caspase-3. This finding particularly occurred in the group which were given prior propolis for 7 days before the induction of anthrax spores compared to the propolis given parallel with anthrax spores injection.

The results of this study showed that high levels of TNF- α were found in rats induced with *B. anthracis* spores on day 14. The discovery is related to the previous study that reported the increase of TNF- α from 300 minutes to the next 7.5 hour after the entry of *B. anthracis* spores into the body, which increased over time, whereas other study showed that TNF- α level peaked at 6 hours and decreased after 24 hours. (5,28)

The mechanism of propolis as an anti-apoptosis has been studied by several previous studies. The presence of CAPE and quercetin in propolis has anti-apoptotic effects that play roles in suppressing T cell activity and inhibiting Nuclear Transcription Factor KappaB (NF- κ B) and IL-2 which stimulates proliferation of T cells, while quercetin can affect the cyclooxygenase pathway.(29,30) The TNF- α levels in the group given propolis for 7 days prior to the induction of anthrax spores had the lowest mean compared to the control group C. These results is similar with previous study that showed the good effectivity of propolis when given for 7 days at a dose of 100 mg/kg.(31) TNF- α is one of the indicators used to detect the existence of an inflammatory process in anthrax infection, and a considerable rise in TNF- α was detected after infection in this investigation.(32,33) Toxins cause the release of pro-inflammatory cytokines like TNF- α , which cause endothelial cell dysfunction and damage to various organs, including the lungs. The mechanism of propolis works against TNF- α is by inhibiting the activation of NF- κ B and the migration of neutrophils, thus lead to decrease in TNF- α production which slow down the release of pro-inflammatory cytokine and prevent progressivity of inflammatory process.(33)

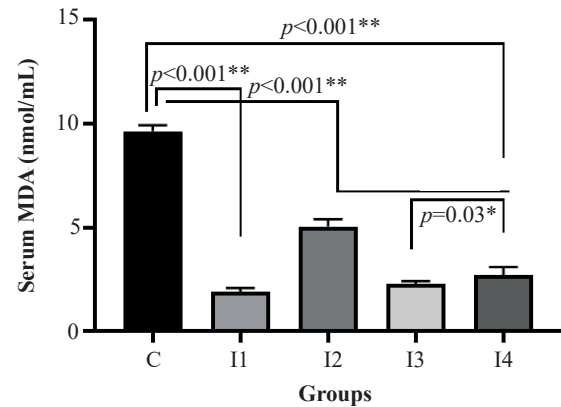


Figure 3. Serum MDA levels in anthrax rats administered with propolis extract. There was a significant difference between groups after tested with ANOVA test and Post-Hoc Tuckey test. ** $p < 0.001$ and * $p < 0.05$ were considered significant.

Propolis administration as a complementary therapy with antibiotic had a lower level of TNF- α compared to the propolis administration alone for 7 days or the propolis administration alone for 14 days. These results showed similar results with the previous study that reported the administration of propolis can reduce TNF- α levels in infection diseases. Previous study documented that there was a significant increase in plasma TNF- α levels in untreated lipopolysaccharide (LPS) mice compared to the control group, whereas CAPE mice injected with LPS showed a significant reduction in TNF- α levels, compared to the untreated LPS mice.(31-33) In this study, groups that were given propolis for 7 days and 14 days showed significant results ($p < 0.05$). This discovery demonstrated that duration of propolis administration after anthrax infection could

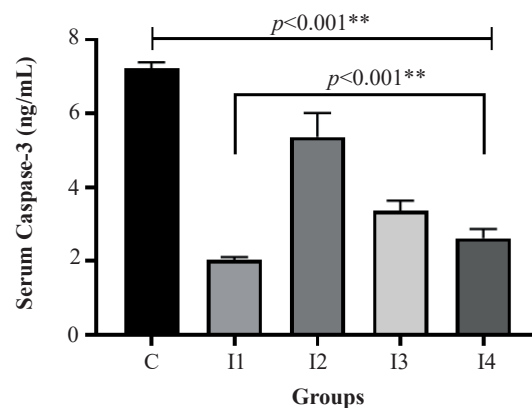


Figure 4. Serum caspase-3 levels in anthrax rats administered with propolis extract. There was a significant difference between groups after tested with ANOVA test. Post-Hoc Tuckey Test also showed significant result within groups. ** $p < 0.001$ and * $p < 0.05$ were considered significant.

contribute to the lower TNF- α levels. Moreover, propolis administration before infection showed better results than groups without propolis administration after exposure to anthrax spores or no propolis. These results are consistent with the study which proved that propolis administration had effect on reducing TNF- α levels significantly.(34)

Propolis had potential mechanism in lowering MDA levels by its antioxidant activity.(35) The antioxidant activity of propolis is based on its content such as flavonoid, quercetin, phenolic acid, and terpenoid.(35,36) MDA is formed by the breakdown of fatty acid in lipid peroxidation.(37) Propolis inhibits ROS which plays role in oxidative stress.(28) The inhibition of oxidative stress results in inhibiting lipid peroxidation process which lowering MDA levels.(38) These results showed that propolis could decrease serum MDA concentration in anthrax rats which related with the previous study that reported MDA levels was reduced in cutaneous anthrax model rats which received 200 mg of propolis ($p<0.05$). (28) Another previous study also showed that 200 mg of propolis reduced the MDA levels more significantly than 100 mg of propolis extracts.(38)

The addition of antibiotics as standard therapy resulted in lower caspase-3 levels compared to using propolis alone. This result related with the previous study that reported caspase-3 was reduced by 1.52 times when CAPE administered via intra-peritoneal before kidney ischemia occurred.(39) The higher the caspase-3 levels will increase the occurrence of apoptosis which in turn will cause failure of several organ systems.(40) Caspase-3 levels reduced significantly ($p<0.05$) in the group that was given propolis alone. This is in accordance with several studies on the effect of propolis on caspase-3, namely that there will be a decrease in caspase-3 in infection (exotoxicity due to clostridial acid) for 3 days.(40) This study proved that the administration of propolis combined with standard antibiotics was superior in reducing caspase-3 levels compared to propolis alone for 14 days, whereas if given only seven days after exposure to infection, the results of caspase reduction were not too significant, but still have an effect if compared to the group without giving propolis or antibiotics.

However, the limitations of this study were not using varying dosage of propolis to investigate the optimal dose to produce maximum effect of its potential anti-oxidant and anti-apoptotic mechanism. Also, other parameters which may affect another inflammatory pathway have not been investigated in the study.

At the cellular level, a new concept can be developed, namely the propolis extract from Gunung Lawu, which has

demonstrated to be an anti-inflammatory and antioxidant in preventing the appearance of multiple organ dysfunction syndrome in the liver, kidney, and lung of mice produced by the spore *B. anthracis*.

Conclusion

This study proved that administration of Indonesian ethanolic extract of propolis significantly lower the TNF- α , caspase-3, and MDA levels. The diminishment of these markers established the effects of Indonesian ethanolic extract of propolis as anti-apoptotic and antioxidant. Thus, these findings could imply the potential action of propolis as a complementary therapy in anthrax. Finally, further research is still needed to analyze other effects of Indonesian ethanolic extract of propolis as antibacterial and antitoxin against anthrax.

Authors Contribution

RTKD and DR were involved in planning and supervised the work, DR and DHP performed the measurements. AS and EH processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. AS and IN performed the parameter calculations and statistical analysis. RTKD, DR, and BP aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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